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(54) Title: GROWTH FACTOR COMPLEX

(57) Abstract: An isolated protein complex is provided which includes a growth factor, growth factor binding protein and vitronectin. Preferably, the isolated protein complex includes an insulin-like growth factor-I, insulin-like growth factor binding protein-3 or insulin-like growth factor binding protein-5 and vitronectin. Also provided are methods of modulating cell proliferation and/or migration by administering said protein complex for the purposes of wound healing, skin repair and tissue replacement therapy. Conversely, by using agents that disrupt growth factor protein complexes formed *in vivo*, growth factor-driven cell proliferation and/or migration may be suppressed such as for the purposes of treating cancers, psoriasis, atherosclerosis and wounds prone to hypertrophic scarring.



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TITLE

## GROWTH FACTOR COMPLEX

FIELD OF THE INVENTION

THIS INVENTION relates to an isolated protein complex which includes a growth factor binding protein and vitronectin. In particular, this invention relates to an isolated protein complex which includes an insulin-like growth factor, an insulin-like growth factor binding protein and vitronectin. Also provided by the invention are growth factor complexes comprising variant growth factors and/or growth factor binding proteins that facilitate or enhance formation of the growth factor complexes. This invention also provides methods of modulating cell proliferation and/or migration by administering said protein complex for the purposes of wound healing, skin repair, cosmetic skin maintenance and tissue replacement therapy. Conversely, by disrupting protein complexes formed *in vivo*, growth factor-driven cell proliferation and/or migration can be suppressed such as for the purposes of treating cancers, psoriasis, atherosclerosis and wounds prone to hypertrophic scarring. These treatments may have medical and veterinary applications.

BACKGROUND OF THE INVENTION

Skin growth, repair and healing are subject to complex biological control mechanisms which act via both positive and negative signals. Such signals act at the level of controlling cell proliferation, differentiation and migration, and are typically mediated by growth factor polypeptides. In this regard, important growth factors include epidermal growth factor (EGF) and insulin-like growth factors (IGF- I and -II).

Human IGF-I has been reported to exert a wide range of biological activities including stimulation of cell proliferation, differentiation and migration, protection from protein degradation and apoptosis, as well as regulation of endocrine factors such as growth hormone. IGF-II has similar properties to IGF-I but appears to be more relevant to carcinogenesis and fetal and embryonic development, IGF-I having a greater role in postnatal development.

Both IGF-I and IGF-II act through a binding interaction with the type I IGF receptor (IGFR). The availability of the IGFs for such an interaction is

regulated by insulin like growth factor binding proteins (IGFBPs 1-6). IGFBPs are known to both positively and negatively regulate IGF function as well as exhibit IGF-independent activity.

Another functional component of IGF pathways is the type II IGFR which is also known as the cation-independent mannose-6-phosphate receptor (CI-MPR). The type II IGFR is a multifunctional protein that binds lysosomal enzymes bearing mannose-6-phosphate moieties, as well as IGF-II, although the functional significance of IGF-II binding is unclear (O'Dell & Day, 1998, *Int. J. Biochem. Cell Biol.* **30** 767; Braulke, 1999, *Horm. Metab. Res.* **31** 242; Nykjaer *et al.*, 1998, *J. Cell. Biol.* **141** 815).

The IGFs have also been reported to bind another group of proteins termed "IGFBP-related proteins" which share structural similarity and include connective tissue growth factor (CTGF) and products encoded by the *mac25*, *nov* and *cyr61* genes. These bind IGFs with much lower affinity than do IGFBPs.

More recently, vitronectin (VN) has been identified as an extracellular matrix protein, structurally unrelated to IGFBPs and IGFBP-related proteins, that binds IGF-II but not IGF-I (Upton *et al.*, 1999, *Endocrinol.* **140** 2928).

Vitronectin is an ~75kD, glycosylated extracellular matrix protein which is also found in blood, and has been implicated in cancers, bone diseases and pathological disorders involving angiogenesis (reviewed in Schwartz *et al.*, 1999, *Int. J. Biochem. Cell Biol.* **31** 539). The role of vitronectin in events such as angiogenesis and tumorigenesis at least partly resides in the ability of vitronectin to bind integrins and to interact with components of the urokinase plasminogen activator system (for example PAI-1, uPAR, plasminogen) to thereby promote cell proliferation, adhesion, spreading and migration. Vitronectin has more specifically been implicated in preventing tumor cell apoptosis in response to drug treatment (Uhm *et al.*, 1999, *Clin. Cancer Res.* **5** 1587). Vitronectin appears to be a carrier of IGF-II in the circulation (McMurtry *et al.*, 1996, *J. Endocrinol.* **150** 149).

#### OBJECT OF THE INVENTION

The present inventor has surprisingly discovered that IGFBPs bind vitronectin, and that IGF-1 can bind vitronectin when bound to an IGFBP.

It is therefore an object of the invention to provide an isolated IGFBP and vitronectin-containing complex.

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#### SUMMARY OF THE INVENTION

In a first aspect, the invention provides an isolated polypeptide complex comprising a growth factor binding protein and vitronectin.

Preferably, the isolated polypeptide complex further comprises a growth factor.

10

A preferred growth factor is insulin-like growth factor-I (IGF-I).

Other growth factors include epidermal growth factor (EGF), fibroblast growth factor (FGF), basic fibroblast growth factor (bFGF), osteopontin, thrombospondin-1, tenascin-C, PAI-1, plasminogen, fibrinogen, fibrin and transferrin.

15

A preferred growth factor binding protein is an insulin-like growth factor binding protein selected from the group consisting of IGFBP1, IGFBP2, IGFBP3, IGFBP4, IGFBP5 and IGFBP6.

A more preferred insulin-like growth factor binding protein is IGFBP2, IGFBP3, IGFBP4 or IGFBP5.

20

An even more preferred insulin-like growth factor binding protein is IGFBP3 or IGFBP5.

In a second aspect, the invention provides an isolated protein complex comprising an IGFBP-related protein and vitronectin.

Preferably, the isolated polypeptide complex further comprises a growth factor, preferably IGF-I.

25

In one embodiment, the IGFBP-related protein is selected from the group consisting of connective tissue growth factor (CTGF), a polypeptide encoded by the *mac25* gene, a polypeptide encoded by the *nov* gene and a polypeptide encoded by the *cyr61* gene.

30

In a third aspect, the invention provides a isolated protein complex comprising vitronectin, a variant growth factor and/or a variant growth factor binding protein.

In one embodiment, the isolated protein complex of this aspect comprises vitronectin and a variant growth factor engineered to include a heparin binding domain (HBD).

5 In another embodiment, the isolated protein complex of this aspect comprises vitronectin and a non-glycosylated growth factor binding protein.

In yet another embodiment, the isolated protein complex of this aspect comprises vitronectin and a variant growth factor selected from the group consisting of des(1-6)IGF-II and des(1-3)IGF-I.

10 Also contemplated according to the first- second- and third- mentioned aspects is that the isolated protein complex of the invention may further include an acid-labile subunit, a polypeptide which can complex with an IGFBP, referred to hereinafter as ALS.

15 The invention according to the first-, second- and third-mentioned aspects also contemplates isolated protein complexes comprising variants and biologically-active fragments of growth factors, growth factor binding proteins, IGFBP-related proteins and vitronectin, and use of such complexes. Biologically-active fragments and variants include within their scope analogues, mutants, agonists and antagonists of said growth factors, growth factor binding proteins, IGFBP-related proteins and vitronectin.

20 In a fourth aspect, the invention provides a pharmaceutical composition comprising one or more isolated protein complexes according to the first-, second- or third-mentioned aspect and a pharmaceutically-acceptable carrier or diluent.

25 In a fifth aspect, the invention provides a pharmaceutical composition comprising a expression construct comprising one or more nucleic acids encoding an isolated protein complex according to the first-, second- or third-mentioned aspect and a pharmaceutically-acceptable carrier or diluent.

30 In a sixth aspect, the invention provides a transformed cell capable of expressing a recombinant protein complex, or recombinant proteins capable of forming said complex, according to the first-, second- or third-mentioned aspects.

In a seventh aspect, the invention provides a method of modulating cell proliferation and/or migration including the step of administering to an animal

or isolated cells thereof, an isolated protein complex according to the first-, second- or third-mentioned aspects.

Preferably, the isolated protein complex comprises an IGFBP and vitronectin.

5 Preferably, the isolated protein complex further comprises IGF-I.

In an eighth aspect, the invention provides a method of modulating cell proliferation and/or migration, including the step of administering to an animal or isolated cells thereof an agent which prevents or disrupts formation of a protein complex according to the first-, second- or third-mentioned aspects.

10 Preferably, the agent prevents or disrupts an interaction between an IGFBP and vitronectin.

More preferably, the agent prevents or disrupts an interaction between IGFBP and vitronectin wherein the protein complex comprises IGF-I.

15 The agent may be an antagonist of an interaction between an IGFBP and vitronectin or between an IGFBP-related protein and vitronectin, for example.

An example of an agent that inhibits formation of IGFBP and vitronectin complexes is IGF-II.

20 As will be described in more detail hereinafter, disruption of interactions between vitronectin and IGFBPs may not only inhibit tumour cell proliferation, but also inhibit tumour metastasis, both events being central to tumour pathology.

25 Further aspects of the invention provide uses of the isolated protein complexes and methods according to the aforementioned aspects of the invention in therapeutic or prophylactic treatments of diseases involving epithelial cells such as psoriasis, atherosclerosis, deterioration of the gastrointestinal epithelium and epithelial breast cancer, and/or in treatments which promote wound healing, skin repair, ulcer and burn healing, *in vitro* skin regeneration such as for grafting of autologous skin, bone regeneration and repair of damaged neuronal tissue.

30 Accordingly, the invention also provides a surgical implant or prosthesis comprising an isolated protein complex of the invention. The surgical implant or prosthesis may be coated, impregnated or otherwise pretreated with

said isolated protein complex.

The animal treated according to the invention may be a mammal, preferably a human, or may be a non-mammalian vertebrate such as a fish, reptile or bird, or cells isolated from any of these.

5                    Throughout this specification, unless otherwise indicated, “comprise”, “comprises” and “comprising” are used inclusively rather than exclusively, so that a stated integer or group of integers may include one or more other non-stated integers or groups of integers.

#### BRIEF DESCRIPTION OF THE FIGURES AND TABLES

10        TABLE 1:     List of references disclosing nucleic acids encoding growth factors and growth factor binding proteins.

TABLE 2:     IGF-I and IGFBP-5 bound to vitronectin stimulates protein synthesis in HaCAT human keratinocytes. Data are derived from measurements of <sup>3</sup>H-leucine incorporation and are expressed as % stimulation above control  
15        (without IGF-I, IGFBP-5 or vitronectin) over 24 hr from a single experiment in which each treatment was tested in triplicate. IGF-I was added to IGFBP-5 (5 ng/well) in the presence (+) of vitronectin (300 ng/well) or in the absence (-) of vitronectin.

FIG. 1:            Competition binding assay using increasing concentrations of  
20        either insulin (▲) or IGF-II (◆) to compete with [<sup>125</sup>I]-IGF-II for binding to 300 ng vitronectin (VN) per well. Radiolabeled IGF-II (10,000 cpm) was added to VN-coated wells and the number of counts bound was determined after overnight incubation and several washes. The binding is expressed as a percentage of the binding observed in control wells with no IGF-II or insulin added. The results are  
25        shown as the average of three replicates ± standard deviation from a representative of three experiments.

FIG. 2:            Competition binding assay using increasing concentrations of  
either IGF-I or IGF-II to compete with [<sup>125</sup>I]-IGF-II for binding to 300 ng  
vitronectin (VN) per well. Radiolabeled IGF-II (10,000 cpm) was added to either  
30        VN (■) - or IGFBP2 (◆)-coated wells and the number of counts bound was determined after overnight incubation and several washes. The binding is expressed as a percentage of the binding observed in control wells with no IGF

added.

FIG. 3: Competition binding assay using increasing concentrations of either proIGF-II (■) or IGF-II (◆) to compete with [<sup>125</sup>I]-IGF-II for binding to 300 ng vitronectin (VN) per well. Radiolabeled IGF-II (10,000 cpm) was added to vitronectin-coated wells and the number of counts bound determined after an overnight incubation and several washes. The binding is expressed as a percentage of the binding observed in the control wells with no added IGF-II or proIGF-II. The results are shown as the average of three replicates ± SEM from two separate experiments.

FIG. 4: Competition binding assay using increasing concentrations of either PAI-1 (■) or IGF-II (◆) to compete with [<sup>125</sup>I]-IGF-II for binding to 300 ng of vitronectin (VN) per well. Radiolabeled IGF-II (10,000 cpm) was added to vitronectin-coated well and the number of counts bound was determined after an overnight incubation and several washes. The binding is expressed as a percentage of the binding observed in the control wells with no added IGF-II or PAI-1. The results are shown as the average of three replicates ± standard deviation from a representative of three separate experiments.

FIG. 5: Competition binding assay comparing effect of preincubation of (A) IGF-II and IGFBP3 and (B) IGF-I and IGFBP3 upon binding to vitronectin. IGFBP3 was non-glycosylated and produced in *E. coli*. Increasing concentrations of IGFBP3 plus either 10,000 cpm [<sup>125</sup>I]-IGF-II (A) or [<sup>125</sup>I]-IGF-I (B) were preincubated for 4 hr and added to vitronectin-coated wells. Alternatively, IGFBP3 plus either 10,000 cpm [<sup>125</sup>I]-IGF-II (A) or [<sup>125</sup>I]-IGF-I (B) were added to vitronectin-coated wells without preincubation. The binding is expressed as the cpm obtained in the absence of non-specific binding. The results are shown as the average of three replicates from a representative of three separate experiments.

FIG. 6: Binding of labelled IGF-I to VN-coated wells in the presence of: (A) IGFBP1, (B) IGFBP2, (C) IGFBP3, (D) IGFBP4, (E) IGFBP5 and (F) IGFBP6. The recombinant IGFBPs were produced in mammalian cells. The data, expressed as average cpm of labelled IGF-I bound/VN-coated well (300 ng/well) in the presence of the indicated IGFBP are from six individual determinations. Ten thousand cpm of radiolabelled IGF-I was added to each well.



FIG. 7: Binding of labelled IGF-I to VN in the presence of "Gly IGFBP-3" (glycosylated IGFBP-3), "IGFBP-3 HBD mutant" (IGFBP-3 with the putative heparin binding domain mutated) and to "non-gly IGFBP-3" (non-glycosylated IGFBP-3).

5 FIG 8: Competition binding assay using increasing concentrations of IGFs and desIGFs to compete with non-glycosylated IGFBP3 incubated with [<sup>125</sup>I]-IGF-I or -II. 30 ng (A) or 10 ng (B) of IGFBP3 plus 10,000 cpm [<sup>125</sup>I]-IGF-II (A) or IGF-I (B) were added to VN-coated wells. Increasing concentrations of IGF-I, IGF-II, des (1-3) IGF-I (not shown) and des(1-6) IGF-II were added to compete  
10 with the radiolabel for binding to VN. Control wells coated with VN were treated with either 10 ng (A) or 30ng (B) IGFBP3 are shown together with control wells coated with VN alone. The binding is expressed as cpm obtained in the absence of non-specific binding. The results are expressed as the average of three replicates ± standard deviation from a representative of three separate experiments.

15 FIG. 9: Stimulation of protein synthesis in human keratinocytes. The data, expressed as % stimulation above control (-VN, -IGF-II) over 24 h is pooled from three replicate experiments in which each treatment was tested in triplicate. The theoretical additive effect represented by open bars (effect of VN alone) combined with grey bars (effect of IGF-II alone) is compared with the actual observed effect  
20 of IGF-II prebound to VN (black bars). In all instances except the lowest concentration of IGF-II tested, the actual observed effect is significantly greater ( $p < 0.05$ ) than the calculated additive effect.

FIG. 10: Binding of labelled IGF-II to VN-coated wells in the presence of: (A) IGFBP1, (B) IGFBP2, (C) IGFBP3, (D) IGFBP4, (E) IGFBP5 and (F)  
25 IGFBP6. The recombinant IGFBPs were produced in mammalian cells. The data, expressed as average cpm of labelled IGF-II bound/VN-coated well (300 ng/well) in the presence of the indicated IGFBP are from six individual determinations. Ten thousand cpm of radiolabelled IGF-II was added to each well.

#### DETAILED DESCRIPTION OF THE INVENTION

30 The present invention has arisen, at least in part, from the discovery by the present inventors that IGF-I binds vitronectin via a binding interaction between an IGFBP and vitronectin. Furthermore, the present invention

describes variant IGFs and IGFBPs that may be used to augment or diminish binding between IGFS, IGFBPs and vitronectin. These discoveries have led the present inventors to manipulate these binding interactions *in vitro* with a view to manipulating contingent *in vivo* biological events associated with cell growth, proliferation and migration. This invention therefore has utility in medical treatments such as wound healing, skin repair and maintenance, bone regeneration, atherosclerosis and cancer therapy in both medical and veterinary areas.

For the purposes of this invention, by "*isolated*" is meant removed from a natural state or otherwise subjected to human manipulation. Isolated material may be substantially or essentially free from components that normally accompany it in its natural state, or may be manipulated so as to be in an artificial state together with components that normally accompany it in its natural state.

By "*polypeptide*" is also meant "*protein*", either term referring to an amino acid polymer.

Proteins and peptides inclusive of growth factor, growth factor binding proteins and vitronectin proteins may be isolated in native, chemical synthetic or recombinant synthetic form.

A "*peptide*" is a protein having no more than fifty (50) amino acids.

A "*biologically-active fragment*" is a fragment, portion or segment of a protein which displays at least 1%, preferably at least 10%, more preferably at least 25% and even more preferably at least 50% of the biological activity of the protein.

Peptides may be readily synthesized by recombinant or chemical synthesis techniques. For example, reference may be made to solution synthesis or solid phase synthesis as described, for example, in Chapter 9 entitled "*Peptide Synthesis*" by Atherton and Shephard which is included in a publication entitled "*Synthetic Vaccines*" edited by Nicholson and published by Blackwell Scientific Publications. Peptide synthesis methods are also described in Chapter 18 of CURRENT PROTOCOLS IN PROTEIN SCIENCE Eds. Coligan *et al.* (John Wiley & Sons NY, 1997) which is incorporated herein by reference.

Alternatively, peptides can be produced by digestion of a polypeptide of the invention with proteinases such as endoLys-C, endoArg-C, endoGlu-C and staphylococcus V8-protease. The digested fragments can be purified by, for example, high performance liquid chromatographic (HPLC) techniques.

5 In a preferred form, the invention provides an isolated protein complex comprising vitronectin, a growth factor and a growth factor binding protein.

By "*growth factor*" is meant a molecule that stimulates or promotes growth of an organism or cells of said organism. A preferred growth factor is a protein or peptide that stimulates cell division, and in particular, mammalian cell division.

Preferably, isolated protein complexes of the invention comprise the growth factor IGF-I.

15 Isolated IGF and IGFBP polypeptides are commercially available from sources such as GroPep (Adelaide, Australia), while VN polypeptides are commercially available from sources such as Promega Corporation (Madison WI USA). Recombinant IGFs, IGFBPs and VN are readily made by persons skilled in the art, as will be discussed in more detail hereinafter.

20 As will be appreciated by the skilled person, the invention also includes precursor forms of IGFs. Examples of pro-IGF-I proteins are IGF-I proteins that have had the signal peptide removed but are not fully processed by cleavage of the E-domain. IGF-I has three different E domains that result from differential mRNA splicing and these precursor proteins may be present in isolated protein complexes of the invention.

25 However, the invention also contemplates isolated protein complexes comprising a growth factor such as epidermal growth factor (EGF; (Heldin *et al.*, 1981, Science **4** 1122-1123), fibroblast growth factor (FGF; Nurcombe *et al.*, 2000, J. Biol. Chem. **275** 30009-30018), basic fibroblast growth factor (bFGF; Taraboletti *et al.*, 1997, Cell Growth. Differ. **8** 471-479),  
30 osteopontin (Nam *et al.*, 2000, Endocrinol. **141** 1100), thrombospondin-1 (Nam *et al.*, 2000, *supra*), tenascin-C (Arai *et al.*, 1996, J. Biol. Chem. **271** 6099), PAI-1

(Nam *et al.*, 1997, Endocrinol. **138** 2972), plasminogen (Campbell *et al.*, 1998, Am. J. Physiol. **275** E321), fibrinogen (Campbell *et al.*, 1999, J. Biol. Chem **274** 30215), fibrin (Campbell *et al.*, 1999, *supra*) or transferrin (Weinzimer *et al.*, 2001, J. Clin. Endocrinol. Metab. **86** 1806).

5                    Preferably, isolated protein complexes comprising osteopontin thrombospondin-1, tenascin-C or PAI-1 further comprise IGFBP-5.

                    Preferably, isolated protein complexes comprising plasminogen fibrinogen, fibrin or transferrin further comprise IGFBP-3.

                    The invention contemplates isolated protein complexes comprising  
10                   monomeric and multimeric vitronectin, as vitronectin can exist in monomeric and multimeric states. In particular, multimeric VN accumulates in areas of vascular injury and also is the predominant form of VN in tissue. Thus the multimeric form of VN provides the opportunity to form a VN complex in which more than one type of growth factor or growth factor binding protein can be delivered at the same  
15                   time.

                    It will also be appreciated by the skilled person that isolated protein complexes of the invention may include vitronectin in "*native*", "*denatured*" or "*extended*" states as are well understood in the art.

                    The invention also contemplates isolated growth factor complexes  
20                   comprising nectinepsin, which is an extracellular matrix protein that shows 60% homology to VN at the amino acid level (Blanchert *et al.*, 1996, J. Biol. Chem. **271** 26220-26226).

                    It will also be understood that variants of growth factors, growth factor binding proteins and/or vitronectin may be used to form isolated protein  
25                   complexes of the invention and may be useful in the methods of use set forth herein.

                    As used herein, "*variant*" proteins, polypeptides and peptides of the invention include those in which one or more amino acids have been replaced by different amino acids .

30                   It is well understood in the art that some amino acids may be changed to others with broadly similar properties without changing the nature of

the activity of the polypeptide (conservative substitutions).

Substantial changes in function may be made by selecting substitutions that are less conservative. Other replacements would be non-conservative substitutions and relatively fewer of these may be tolerated.

5 Generally, the substitutions which are likely to produce the greatest changes in a polypeptide's properties are those in which (a) a hydrophilic residue (*e.g.*, Ser or Thr) is substituted for, or by, a hydrophobic residue (*e.g.*, Ala, Leu, Ile, Phe or Val); (b) a cysteine or proline is substituted for, or by, any other residue; (c) a residue having an electropositive side chain (*e.g.*, Arg, His or Lys) is substituted

10 for, or by, an electronegative residue (*e.g.*, Glu or Asp) or (d) a residue having a bulky side chain (*e.g.*, Phe or Trp) is substituted for, or by, one having a smaller side chain (*e.g.*, Ala, Ser) or no side chain (*e.g.*, Gly).

Variants also include proteins, polypeptides and peptides which have been altered, for example by conjugation or complexing with other chemical

15 moieties or by post-translational modification techniques as would be understood in the art. Such derivatives include amino acid deletions and/or additions.

Other polypeptide and peptide variants contemplated by the invention include, but are not limited to, modification to side chains, incorporation of unnatural amino acids and/or their derivatives during peptide, polypeptide or

20 protein synthesis and the use of crosslinkers and other chemicals which impose conformational constraints on the polypeptides and peptide variants of the invention. Examples of side chain modifications contemplated by the present invention include modifications of amino groups such as by acylation with acetic anhydride; acylation of amino groups with succinic anhydride and

25 tetrahydrophthalic anhydride; amidination with methylacetimidate; carbamoylation of amino groups with cyanate; pyridoxylation of lysine with pyridoxal-5-phosphate followed by reduction with NaBH<sub>4</sub>; reductive alkylation by reaction with an aldehyde followed by reduction with NaBH<sub>4</sub>; and trinitrobenzylation of amino groups with 2, 4, 6-trinitrobenzene sulphonic acid

30 (TNBS).

The carboxyl group may be modified by carbodiimide activation via *O*-acylisourea formation followed by subsequent derivitization, by way of

example, to a corresponding amide.

The guanidine group of arginine residues may be modified by formation of heterocyclic condensation products with reagents such as 2,3-butanedione, phenylglyoxal and glyoxal.

5 Sulphydryl groups may be modified by methods such as performic acid oxidation to cysteic acid; formation of mercurial derivatives using 4-chloromercuriphenylsulphonic acid, 4-chloromercuribenzoate; 2-chloromercuri-4-nitrophenol, phenylmercury chloride, and other mercurials; formation of a mixed disulphides with other thiol compounds; reaction with maleimide, maleic  
10 anhydride or other substituted maleimide; carboxymethylation with iodoacetic acid or iodoacetamide; and carbamoylation with cyanate at alkaline pH.

Tryptophan residues may be modified, for example, by alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or sulphonyl halides or by oxidation with N-bromosuccinimide.

15 Tyrosine residues may be modified by nitration with tetranitromethane to form a 3-nitrotyrosine derivative.

The imidazole ring of a histidine residue may be modified by N-carbethoxylation with diethylpyrocarbonate or by alkylation with iodoacetic acid derivatives.

20 Examples of incorporating unnatural amino acids and derivatives during peptide synthesis include but are not limited to, use of 4-amino butyric acid, 6-aminohexanoic acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 4-amino-3-hydroxy-6-methylheptanoic acid, t-butylglycine, norleucine, norvaline, phenylglycine, ornithine, sarcosine, 2-thienyl alanine and/or D-isomers of amino  
25 acids.

Modifications also include within their scope *O*- and *N*- linked glycosylation variants and non-glycosylated forms of proteins that, in their naturally-occurring state, are glycosylated.

30 With regard to variants, these may be created by mutagenizing a polypeptide or by mutagenizing an encoding nucleic acid, such as by random mutagenesis or site-directed mutagenesis. Examples of nucleic acid mutagenesis methods are provided in in Chapter 9 of CURRENT PROTOCOLS IN

MOLECULAR BIOLOGY, Ausubel *et al.*, *supra* which is incorporated herein by reference.

It will be appreciated by the skilled person that site-directed mutagenesis is best performed where knowledge of the amino acid residues that contribute to biological activity is available. In many cases, this information is not available, or can only be inferred by molecular modelling approximations, for example.

In such cases, random mutagenesis is contemplated. Random mutagenesis methods include chemical modification of proteins by hydroxylamine (Ruan *et al.*, 1997, *Gene* **188** 35), incorporation of dNTP analogs into nucleic acids (Zaccolo *et al.*, 1996, *J. Mol. Biol.* **255** 589) and PCR-based random mutagenesis such as described in Stemmer, 1994, *Proc. Natl. Acad. Sci. USA* **91** 10747 or Shafikhani *et al.*, 1997, *Biotechniques* **23** 304, each of which references is incorporated herein. It is also noted that PCR-based random mutagenesis kits are commercially available, such as the Diversify™ kit (Clontech).

The invention also contemplates use of growth factor variants such as des(1-6)IGF-II and des(1-3)IGF-I to form isolated protein complexes of the invention.

Other variant IGFs and IGFBPs useful as agonists or antagonists will be described in more detail hereinafter.

#### ***Recombinant growth factor complexes***

It will be appreciated that isolated protein complexes may be produced using recombinant growth factors, growth factor binding proteins and/or vitronectin, by expression of an encoding nucleic acid in an appropriate host cell or in a cell-free expression system as are well known in the art.

The term “*nucleic acid*” as used herein designates single-or double-stranded mRNA, RNA, cRNA and DNA, said DNA inclusive of cDNA and genomic DNA.

A “*polynucleotide*” is a nucleic acid having eighty (80) or more contiguous nucleotides, while an “*oligonucleotide*” has less than eighty (80) contiguous nucleotides.

A “probe” may be a single or double-stranded oligonucleotide or polynucleotide, suitably labeled for the purpose of detecting complementary sequences in Northern or Southern blotting, for example.

5 A “primer” is usually a single-stranded oligonucleotide, preferably having 15-50 contiguous nucleotides, which is capable of annealing to a complementary nucleic acid “template” and being extended in a template-dependent fashion by the action of a DNA polymerase such as *Taq* polymerase, RNA-dependent DNA polymerase or Sequenase™.

10 Nucleic acids encoding IGFs, EGF, IGFBPs, ALS and VN are well known in the art and have been available for many years. However, the skilled person is referred to Table 1 which lists references that provide examples of these nucleic acid sequences. All references in Table 1 are incorporated herein by reference.

15 Nucleic acids useful according to the present invention may be prepared according to the following procedure:

- (i) creating primers which are, optionally, degenerate wherein each comprises a respective portion of a target nucleic acid; and
- (ii) using said primers in combination with a nucleic acid  
20 amplification technique to amplify one or more amplification products from a nucleic acid extract.

Suitable nucleic acid amplification techniques are well known to the skilled person, and include polymerase chain reaction (PCR) as for example described in Chapter 15 of Ausubel *et al. supra*, which is incorporated herein by  
25 reference; strand displacement amplification (SDA) as for example described in U.S. Patent No 5,422,252 which is incorporated herein by reference; rolling circle replication (RCR) as for example described in Liu *et al.*, 1996, J. Am. Chem. Soc. 118 1587, International application WO 92/01813 and International Application WO 97/19193 which are incorporated herein by reference; nucleic acid sequence-  
30 based amplification (NASBA) as for example described by Sooknanan *et al.*, 1994, Biotechniques 17 1077 which is incorporated herein by reference; ligase chain



reaction (LCR) as for example described in International Application WO89/09385 which is incorporated by reference herein; and Q- $\beta$  replicase amplification as for example described by Tyagi *et al.*, 1996, Proc. Natl. Acad. Sci. USA **93** 5395 which is incorporated herein by reference.

5                   As used herein, an “*amplification product*” refers to a nucleic acid product generated by nucleic acid amplification techniques.

Recombinant proteins may be prepared by any suitable procedure known to those of skill in the art.

For example, the recombinant protein may be prepared by a  
10                   procedure including the steps of:

- (i)       preparing an expression construct which comprises a nucleic acid, operably linked to one or more regulatory nucleotide sequences;
- (ii)      transfecting or transforming a suitable host cell with the  
15                   expression construct; and
- (iii)     expressing the polypeptide in said host cell.

For the purposes of host cell expression, the recombinant nucleic acid is operably linked to one or more regulatory sequences in an expression vector.

20                   An “*expression vector*” may be either a self-replicating extra-chromosomal vector such as a plasmid, or a vector that integrates into a host genome.

By “*operably linked*” is meant that said regulatory nucleotide sequence(s) is/are positioned relative to the recombinant nucleic acid of the  
25                   invention to initiate, regulate or otherwise control transcription.

Regulatory nucleotide sequences will generally be appropriate for the host cell used for expression. Numerous types of appropriate expression vectors and suitable regulatory sequences are known in the art for a variety of host cells.

30                   Typically, said one or more regulatory nucleotide sequences may include, but are not limited to, promoter sequences, leader or signal sequences,

ribosomal binding sites, transcriptional start and termination sequences, translational start and termination sequences, and enhancer or activator sequences.

Constitutive or inducible promoters as known in the art are contemplated by the invention. The promoters may be either naturally occurring  
5 promoters, or hybrid promoters that combine elements of more than one promoter.

In a preferred embodiment, the expression vector contains a selectable marker gene to allow the selection of transformed host cells. Selectable marker genes are well known in the art and will vary with the host cell used.

The expression vector may also include a fusion partner (typically  
10 provided by the expression vector) so that the recombinant polypeptide of the invention is expressed as a fusion polypeptide with said fusion partner. The main advantage of fusion partners are that they assist identification and/or purification of said fusion polypeptide and also enhance protein expression levels and overall yield.

15 In order to express said fusion polypeptide, it is necessary to ligate a nucleotide sequence according to the invention into the expression vector so that the translational reading frames of the fusion partner and the nucleotide sequence of the invention coincide.

Well known examples of fusion partners include, but are not  
20 limited to, glutathione-S-transferase (GST), Fc portion of human IgG, maltose binding protein (MBP) and hexahistidine (HIS<sub>6</sub>), which are particularly useful for isolation of the fusion polypeptide by affinity chromatography. For the purposes of fusion polypeptide purification by affinity chromatography, relevant matrices for affinity chromatography are glutathione-, amylose-, and nickel- or cobalt-  
25 conjugated resins respectively. Many such matrices are available in "kit" form, such as the QIAexpress<sup>TM</sup> system (Qiagen) useful with (HIS<sub>6</sub>) fusion partners and the Pharmacia GST purification system.

Another fusion partner well known in the art is green fluorescent protein (GFP). This fusion partner serves as a fluorescent "tag" which allows the  
30 fusion polypeptide of the invention to be identified by fluorescence microscopy or by flow cytometry. The GFP tag is useful when assessing subcellular localization of the fusion polypeptide of the invention, or for isolating cells which express the

fusion polypeptide of the invention. Flow cytometric methods such as fluorescence activated cell sorting (FACS) are particularly useful in this latter application.

In some cases, the fusion partners also have a protease cleavage site, such as for Factor X<sub>a</sub> or Thrombin, which allow the relevant protease to partially digest the fusion polypeptide of the invention and thereby liberate the recombinant polypeptide of the invention therefrom. The liberated polypeptide can then be isolated from the fusion partner by subsequent chromatographic separation.

Fusion partners according to the invention also include within their scope "*epitope tags*", which are usually short peptide sequences for which a specific antibody is available. Well known examples of epitope tags for which specific monoclonal antibodies are readily available include c-myc, influenza virus haemagglutinin and FLAG tags.

The recombinant protein may be conveniently prepared by a person skilled in the art using standard protocols as for example described in Sambrook, *et al.*, MOLECULAR CLONING. A Laboratory Manual (Cold Spring Harbor Press, 1989), incorporated herein by reference, in particular Chapter 16 and 17; CURRENT PROTOCOLS IN MOLECULAR BIOLOGY Eds. Ausubel *et al.*, (John Wiley & Sons, Inc. 1995-1999), incorporated herein by reference, in particular Chapters 10 and 16; and CURRENT PROTOCOLS IN PROTEIN SCIENCE Eds. Coligan *et al.*, (John Wiley & Sons, Inc. 1995-1999) which is incorporated by reference herein, in particular Chapters 1, 5 and 6.

In one embodiment, recombinant expression of growth factor, growth factor binding protein and vitronectin may be performed separately, and complexes formed therefrom.

In another embodiment, recombinant expression of growth factor, growth factor binding protein and vitronectin may be performed in the same cell, and complexes formed therefrom.

As hereinbefore, polypeptides of the invention may be produced by culturing a host cell transformed with said expression construct comprising a nucleic acid encoding a polypeptide, or polypeptide homolog, of the invention.

The conditions appropriate for protein expression will vary with the choice of expression vector and the host cell. This is easily ascertained by one skilled in the art through routine experimentation.

Suitable host cells for recombinant expression include bacteria  
5 such as *E. coli.*, *Clostridium* sp., *Pseudomonas* sp., yeast, plant cells, insect cells (such as *Sf9*) and mammalian cells such as fibroblasts and keratinocytes.

Preferred host cells are human keratinocytes.

Inducible and non-inducible expression vectors are contemplated.  
In stably transfected mammalian cells, a number of inducible and repressible  
10 systems have been devised including metallothionine (MT) inducible and tetracycline and repressible (tetR), each of which is contemplated by the present invention.

Particular examples of suitable expression vectors and methods of recombinant IGFBP expression may be found in United States Patent 5,973,115  
15 which is incorporated herein by reference.

#### ***Mimetics, Agonists and Antagonists***

The invention contemplates agents which may promote, prevent or disrupt formation of protein complexes comprising growth factors, growth factor binding proteins and vitronectin. Such an agent may be a mimetic. The term  
20 "*mimetic*" is used herein to refer to molecules that are designed to resemble particular functional regions of proteins or peptides, and includes within its scope the terms "*agonist*", "*analogue*" and "*antagonist*" as are well understood in the art.

Of relevance is the elucidation of the portions of IGFs and IGFBPs  
25 which are responsible for IGF-IGFBP binding, as described in International Publication WO00/23469. Furthermore, agonist variants of IGF-I have been made which selectively bind IGFBP-1 or IGFBP-3, as described in International Publication WO00/40612.

It is therefore contemplated that agents could be engineered which  
30 disrupt or prevent formation of polypeptide complexes between IGFBPs and VN. An example would be a peptide which competes for binding of the IGFBP to VN by resembling the binding site on VN or the IGFBP.

As will be described in more detail hereinafter, IGF-II is an agent that can inhibit binding between an IGFBP and vitronectin. It is also proposed that residues V<sup>35</sup> or S<sup>36</sup> along with S<sup>39</sup> of the RVSRRSR sequence at positions 34-40 in the C-domain of IGF-II could be mutated to basic residues to thereby resemble the HBD of IGFBP3 (BXBBB wherein B is a basic amino acid residue). This would create an agent even more capable of inhibiting formation of a complex between IGFBPs and vitronectin.

An example of an agonist contemplated by the present invention is IGF-1 engineered to include a heparin binding domain (HBD) of an IGFBP to thereby bind vitronectin directly. For example, the sequence SSSRRAPQT in the C-domain of IGF-I may be engineered to have a BXBBB motif. A preferred BXBBB motif is the KGRKR sequence of IGFBP-3 (residues 228-232).

Alternatively, the putative vitronectin-binding domain of IGF-II: RVSRRSR (residues 34-40) may be introduced into IGF-I.

An example of an antagonist of the invention is an IGFBP engineered to mutate basic residues in the HBD (as hereinbefore described) to reduce or prevent binding of the IGFBP to vitronectin.

Suitably, the engineered IGFBP is capable of binding IGF-I.

Preferably, the engineered IGFBP is IGFBP-3 or IGFBP-5.

It is also contemplated that an analogue of an IGFBP could be engineered which enables formation of a complex between the analogue and VN. Suitably, the analogue would also bind an IGF. Potential advantages of such an analogue is that it might be more readily synthesized or isolated than an IGFBP, have a particular desired biological half-life and perhaps be engineered to specifically bind IGF-I.

The aforementioned mimetics may be peptides, polypeptides or other organic molecules, preferably small organic molecules, with a desired biological activity and half-life.

Computer-assisted structural database searching is becoming increasingly utilized as a procedure for identifying mimetics.. Database searching methods which, in principle, may be suitable for identifying mimetics, may be found in International Publication WO 94/18232 (directed to producing HIV

antigen mimetics), United States Patent No. 5,752,019 and International Publication WO 97/41526 (directed to identifying EPO mimetics), each of which is incorporated herein by reference.

Other methods include a variety of biophysical techniques which indentify molecular interactions. These allow for the screening of candidate molecules according to whether said candidate molecule affects formation of IGF-IGFBP-VN complexes, for example. Methods applicable to potentially useful techniques such as competitive radioligand binding assays (see Upton *et al.*, 1999, *supra* for a relevant method), analytical ultracentrifugation, microcalorimetry, surface plasmon resonance and optical biosensor-based methods are provided in Chapter 20 of CURRENT PROTOCOLS IN PROTEIN SCIENCE Eds. Coligan *et al.*, (John Wiley & Sons, 1997) which is incorporated herein by reference.

#### ***Pharmaceutical compositions***

The invention includes administration of a protein complex of the invention in the form of a pharmaceutical composition. Pharmaceutical compositions of the invention may include isolated protein complexes that comprise variant IGFs and/or IGFBPs or agents that disrupt or prevent formation of said complexes as hereinbefore described.

Suitably, the pharmaceutical composition comprises a pharmaceutically-acceptable carrier. Pharmaceutical compositions may also include polypeptide variants, fragments or mimetics as hereinbefore defined.

By “*pharmaceutically-acceptable carrier*” is meant a solid or liquid filler, diluent or encapsulating substance that may be safely used in systemic administration. Depending upon the particular route of administration, a variety of carriers, well known in the art may be used. These carriers may be selected from a group including sugars, starches, cellulose and its derivatives, malt, gelatine, talc, calcium sulfate, vegetable oils, synthetic oils, polyols, alginic acid, phosphate buffered solutions, emulsifiers, isotonic saline, and pyrogen-free water.

Any suitable route of administration may be employed for providing a patient with the composition of the invention. For example, oral, rectal, parenteral, sublingual, buccal, intravenous, intra-articular, intra-muscular,

intra-dermal, subcutaneous, inhalational, intraocular, intraperitoneal, intracerebroventricular, transdermal and the like may be employed. Intramuscular and subcutaneous injection is appropriate, for example, for administration of immunogenic compositions, vaccines and DNA vaccines.

5 Dosage forms include tablets, dispersions, suspensions, injections, solutions, syrups, troches, capsules, suppositories, aerosols, transdermal patches and the like. These dosage forms may also include injecting or implanting controlled releasing devices designed specifically for this purpose or other forms of implants modified to act additionally in this fashion. Controlled release of the  
10 therapeutic agent may be effected by coating the same, for example, with hydrophobic polymers including acrylic resins, waxes, higher aliphatic alcohols, polylactic and polyglycolic acids and certain cellulose derivatives such as hydroxypropylmethyl cellulose. In addition, the controlled release may be effected by using other polymer matrices, liposomes and/or microspheres.

15 Pharmaceutical compositions of the present invention suitable for oral or parenteral administration may be presented as discrete units such as capsules, sachets or tablets each containing a pre-determined amount of one or more therapeutic agents of the invention, as a powder or granules or as a solution or a suspension in an aqueous liquid, a non-aqueous liquid, an oil-in-water  
20 emulsion or a water-in-oil liquid emulsion.

With regard to pharmaceutical compositions comprising IGF and IGFBPs, particular reference is made to U.S. patent 5,936,064 and International Publications WO99/62536 and WO99/54359 which are incorporated herein by reference.

25 Pharmaceutical compositions of the invention may also include expression vectors such as viral vectors such as vaccinia, and viral vectors useful in gene therapy. The latter include adenovirus and adenovirus-associated viruses (AAV) such as described in Braun-Falco *et al.*, 1999, Gene Ther. 6 432, retroviral and lentiviral vectors such as described in Buchshacher *et al.*, 2000, Blood 95  
30 2499 and vectors derived from herpes simplex virus and cytomegalovirus. A

general overview of viral vectors useful in endocrine gene therapy is provided in Stone *et al.*, 2000, J. Endocrinol. **164** 103.

The present invention may also utilize specific expression vectors which target gene expression to epidermal cells, such as described in United States Patent 5,958,764 and for *in vivo* wound healing applications, such as  
5 described in United States Patent 5,962,427.

Each of the aforementioned publications is incorporated herein by reference.

### ***Therapeutic uses***

10 The invention provides methods of treatment using polypeptide complexes of the invention. These methods are particularly aimed at therapeutic treatment of mammals, and more particularly, humans.

Such methods include administration of pharmaceutical compositions as hereinbefore defined, and may be by way of microneedle  
15 injection into specific tissue sites, such as described in U.S. patent 6,090,790, topical creams, lotions or sealant dressings applied to wounds, burns or ulcers, such as described in U.S. patent 6,054,122 or implants which release the composition such as described in International Publication WO99/47070.

Gene therapy is also applicable in this regard, such as according to  
20 methods set forth in United States Patent 5,929,040 and United States Patent 5,962,427.

There also exist methods by which skin cells can be genetically modified for the purpose of creating skin substitutes, such as by genetically engineering desired growth factor expression (Supp *et al.*, 2000, J. Invest.  
25 Dermatol. **114** 5). An example of a review of this field is provided in Bevan *et al.*, Biotechnol. Gent. Eng. Rev. **16** 231.

Also contemplated is "seeding" a recipient with transfected or transformed cells, such as described in International Publication WO99/11789 .

These methods can be used to stimulate cell proliferation and  
30 thereby facilitate or progress wound and burn healing, repair of skin lesions such as ulcers, tissue replacement and grafting such as by *in vitro* culturing of



autologous skin, re-epithelialization of internal organs such as kidney and lung and repair of damaged nerve tissue.

5 Skin replacement therapy has become well known in the art, and may employ use of co-cultured epithelial/keratinocyte cell lines, for example as described in Kehe *et al.*, 1999, Arch. Dermatol. Res. **291** 600 or *in vitro* culture of primary (usually autologous) epidermal, dermal and/or keratinocyte cells. These techniques may also utilize engineered biomaterials and synthetic polymer “scaffolds”.

10 Examples of reviews of the field in general are provided in Terskikh & Vasiliev, 1999, Int. Rev. Cytol. **188** 41 and Eaglestein & Falanga, 1998, Cutis **62** 1.

15 More particularly, the production of replacement oral mucosa useful in craniofacial surgery is described in Izumi *et al.*, 2000, J. Dent. Res. **79** 798. Fetal keratinocytes and dermal fibroblasts can be expanded *in vitro* to produce skin for grafting to treat skin lesions, such as described in Fauza *et al.*, J. Pediatr. Surg. **33** 357, while skin substitutes from dermal and epidermal skin elements cultured *in vitro* on hyaluronic acid-derived biomaterials have been shown to be potentially useful in the treatment of burns (Zacchi *et al.*, 1998, J. Biomed. Mater. Res. **40** 187).

20 Another aspect of epithelial cell therapy relates to healing of the epithelial lining of the gastrointestinal tract to treat or prevent impaired gut function.

25 Polymer scaffolds are also contemplated for the purpose of facilitating replacement skin engineering, as for example described in Sheridan *et al.*, 2000, J. Control Release **14** 91 and Fauza *et al.*, 1998, *supra*, as are microspheres as agents for the delivery of skin cells to wounds and burns (LaFrance & Armstrong, 1999, Tissue Eng. **5** 153).

30 The aforementioned techniques may be readily utilized according to the present invention by use of isolated protein complexes of the invention to promote skin cell proliferation for the purposes of tissue replacement and for cosmetic skin treatments.

With regard to bone regeneration, the invention provides surgical or prosthetic implants coated, impregnated or otherwise pretreated with an isolated protein complex of the invention.

Conversely, inhibition or suppression of cell proliferation and migration by preventing or disrupting formation of IGF-IGFBP-VN complexes may constitute a prophylactic or therapeutic treatment of psoriasis or malignancies such as epithelial cancers such as breast cancer.

The invention also contemplates a method of differentiating a stem or progenitor cell by administering an isolated protein complex of the invention to said stem or progenitor cell. Isolated protein complexes comprising variant growth factors and IGFBPs may also be applicable to this method.

Differentiated cells produced according to this method may be useful in therapeutic methods such as as hereinbefore described.

For example, smooth muscle cells are considered to be "*mesenchymal stem cells*" and may be driven to differentiate into fibroblasts, stromal cells, endothelial cells, bone or adipocytes.

In order that the invention may be readily understood and put into practical effect, particular preferred embodiments will now be described by way of the following non-limiting examples.

All competitive radioligand binding assays as described herein were performed essentially as described in Upton *et al.*, 1999, *supra*.

### EXAMPLE 1

#### *Competition binding assay to assess ability of insulin, pro-IGF-II and IGF-I to compete with IGF-II for binding to vitronectin*

Due to the similarity in structure shared between IGFs and insulin, binding of insulin to vitronectin was examined. Crosslinking experiments performed by Upton *et al.*, 1999, *supra* indicated that insulin was unlikely to compete with IGF-II for binding to vitronectin, as was the case with IGF-I. As a consequence, high concentrations of insulin were examined in order to establish whether insulin could compete with radiolabelled IGF-II for binding to vitronectin. The results shown in FIG.1 indicated that, as for the situation originally observed with IGF-I

(Upton *et al.*, 1999, *supra*), insulin competed poorly with [ $^{125}$ I]-IGF-II for binding to vitronectin.

Investigation of IGF-I as a competitor for IGF-II binding to vitronectin revealed that IGF-I, could compete with the binding of [ $^{125}$ I]-IGF-II to vitronectin (FIG. 2). However, IGF-I was markedly less effective than IGF-II in competing with radiolabelled IGF-II for binding to vitronectin, requiring an approximate 3000 fold increase in IGF-I concentration above IGF-II to achieve the same effect.

Similar studies demonstrated that proIGF-II could compete with [ $^{125}$ I]-IGF-II for binding to vitronectin (FIG. 3). ProIGF-II was not as effective as IGF-II in competing with [ $^{125}$ I]-IGF-II for binding to vitronectin with IC<sub>50</sub> values of 65.6 nM and 9.6 nM respectively. Thus, the presence of the E domain within proIGF-II represents a structural modification that alters the binding to vitronectin. This could be due to steric hindrance by the additional domain in proIGF-II compared to IGF-II, or may be a result of an altered structure of the proIGF-II molecule that alters the affinity of proIGF-II for vitronectin.

## EXAMPLE 2

### *Investigation of PAI-1 and uPAR as competitors for binding of IGF-II to vitronectin*

PAI-1 and suPAR were investigated as molecules which might compete with IGF-II for binding to vitronectin, as these proteins have been reported to bind vitronectin (Declerck *et al.*, 1988, J. Biol. Chem. **263** 15454; Wei *et al.*, 1994, J. Biol. Chem. **269** 32380; Kanse *et al.*, 1996, Exp. Cell Res. **224** 344). At concentrations up to 2000 nM competition of PAI-1 with  $^{125}$ I-IGF-II for binding to vitronectin was observed with an approximate IC<sub>50</sub> value of 524 nM (FIG. 4). While this concentration is relatively high in terms of IC<sub>50</sub> values, such concentrations can be found *in vivo* associated with tumours (Grondahl-Hansen *et al.*, 1993, Cancer Res. **53** 2513). Indeed, Kjoller *et al.*, 1997, Exp. Cell Res. **232** 420, found 370 nM of PAI-1 was required to achieve half maximal effect in an assay assessing the ability of PAI-1 to inhibit cell migration of WISH cells. This inhibition was postulated to result via PAI-1 competing with integrins and uPAR for binding to vitronectin. Thus, the observed interaction between PAI-I, IGF-II

and vitronectin may indeed have some functional consequences *in vivo*, particularly when IGF-II and PAI-1 levels are highly expressed as is sometimes found in tumours.

While it is known that PAI-1 binds to the N-terminal somatomedin B domain of vitronectin, the high concentrations of PAI-1 required to effectively compete with IGF-II for binding to vitronectin suggested that these studies have not provided any clear information regarding the location of IGF-II of the binding site on vitronectin. However, the results may be interpreted two ways. The first possibility is that the observed competition between IGF-II and PAI-1 is due to partial competition at the primary high affinity site on vitronectin within the somatomedin B domain via steric hindrance. Alternatively, the competition could be due to direct competition between IGF-II and PAI-1, for binding to a site on vitronectin to which PAI-1 binds with a reduced affinity. Further experimental studies are required to clarify this issue.

While the present inventors were unable to show that soluble suPAR competes for binding of IGF-II to VN, this may have been due to that fact that lyophilized suPAR was used after international transportation. There is no evidence that reconstituted suPAR is biologically active after reconstitution, although the observation that [<sup>125</sup>I]-suPAR did not bind VN (data not shown) perhaps supports an interpretation that the suPAR used in these studies was inactive.

Use of similar techniques to those that identified the PAI-1 binding site on vitronectin to be within a fragment of vitronectin containing the 44 N-terminal amino acids (Deng *et al.*, 1995, *Thromb. Haemost.* 74 66), may establish whether the competition observed between PAI-1 and IGF-II for binding to vitronectin is a result of either of these possibilities.

It has previously been shown that the soluble form of the urokinase receptor (suPAR) can bind to immobilized vitronectin (Wei *et al.*, 1994, *supra*). In the studies reported here, however, even at concentrations up to 300 nM, no competition between suPAR and [<sup>125</sup>I]-IGF-II for binding to vitronectin was observed.

### EXAMPLE 3

***Binding of IGF-I and IGF-II to vitronectin in the presence of non-glycosylated recombinant IGFBP-3***

To investigate whether IGFBPs could mediate binding of the IGFs to vitronectin, the effect of increasing concentrations of IGFBP-3 in the presence of [<sup>125</sup>I]-IGF-I or [<sup>125</sup>I]-IGF-II was assessed. The results are shown in FIG. 5.

At the concentrations tested, 10 ng of IGFBP-3 per well caused the greatest amount of [<sup>125</sup>I]-IGF-I to bind to vitronectin coated wells, whereas 30 ng of IGFBP-3 per well caused the greatest amount of [<sup>125</sup>I]-IGF-II to bind to vitronectin coated wells. The effect was most noticeable in the case of [<sup>125</sup>I]-IGF-I, since low counts of [<sup>125</sup>I]-IGF-I bound to vitronectin, and even at the lowest concentration of IGFBP-3 (3.7 ng), this binding was increased (FIG. 5A). Conversely, [<sup>125</sup>I]-IGF-II did not show an increase over binding obtained on vitronectin coated wells alone, until concentrations of 11 ng/100 µL - 33 ng/100 µL were reached (FIG. 5B).

Preincubation of IGFBP-3 with either [<sup>125</sup>I]-IGF-I or [<sup>125</sup>I]-IGF-II did not alter the binding to vitronectin as compared to the non pre-incubated concentrations of IGFBP-3 and [<sup>125</sup>I]-IGF-I or [<sup>125</sup>I]-IGF-II.

**EXAMPLE 4**

***Binding of IGF-I to vitronectin in the presence of recombinant IGFBPs produced in mammalian cells***

Binding assays examining the ability of [<sup>125</sup>I]-IGF-I to bind to VN-coated dishes in the presence of IGFBPs, added at the same time as the radiolabel, were performed as described in Upton *et al.*, 1999, *supra*. The data are shown in FIG. 6. Increasing amounts of IGFBP-2, -4 and -5 resulted in increased binding of labelled IGF-I to vitronectin coated wells. At the highest amount of IGFBP tested, 5 ng, binding of labelled IGF-I was increased approximately 2.8-, 3.8- and 8-fold for IGFBP-2, 4 and 5 compared to control wells where VN, but no IGFBPs, were present.

The presence of IGFBP-3 at 0.05, 0.2 and 0.5 ng/well also increased binding of labelled IGF-I to VN while 2 and 5 ng of IGFBP-3/well appeared to inhibit binding of the radiolabel. All concentrations of IGFBP-1 and -6 tested were also inhibitory.

These results demonstrate that unlike the situation with IGF-II, minimal direct binding of IGF-I to VN is observed. However, the presence of IGFBPs, especially IGFBP-2, -3, -4 and -5 enhances IGF binding to VN-coated wells, suggesting that IGFBPs mediate the binding of IGF-I to VN in this situation. In addition, the data suggests that IGFBPs have the potential to both enhance and inhibit binding of IGF-I to VN, depending on a) which IGFBPs are present and b) the amount of IGFBP present.

#### EXAMPLE 5

##### *Binding of labelled IGF-I to VN in the presence IGFBP-3 variants*

Binding assays examining the ability of [ $^{125}$ I]-IGF-I to bind to VN-coated dishes in the presence of an IGFBP-3 variant in which the putative "heparin binding domain" was mutated (IGFBP-3 HBD) and a variant in which glycosylation sites had been mutated (Non-gly IGFBP-3) were performed as described above. The data are shown in FIG. 7.

The IGFBP-3 glycosylation mutant used in these binding studies had three potential *O*-glycosylation sites at positions Asn89, Asn109, Asn172 mutated to Ala.

The IGFBP-3 HBD mutant did not enhance binding of [ $^{125}$ I]-IGF-I to VN-coated dishes, suggesting the heparin binding domain of IGFBP-3 is involved in binding IGFBP-3 to VN. Other studies have identified that amino acid residues outside of the putative "heparin binding domain" are responsible for IGF-I binding to IGFBP-3, hence it is most likely that the results represent decreased binding of IGFBP-3 to VN, rather than decreased binding of labelled IGF-I to IGFBP-3 (Imai *et al.*, 2000, J Biol Chem **275**:18188-18194).

Interestingly, the non-glycosylated IGFBP-3 mutant significantly enhanced binding of [ $^{125}$ I]-IGF-I to bind to VN-coated dishes. The striking 20-fold increase in binding of labelled IGF-I to VN-coated wells in the presence of 2 ng of the mutant IGFBP-3 is intriguing and suggests that glycosylation of IGFBP-3 inhibits interaction of either a) IGF-I to IGFBP-3 or b) IGFBP-3 with VN. Alternatively, both interactions may be hindered by the presence of carbohydrates.

Whether non-glycosylated IGFBP-3 is functionally relevant *in vivo* remains to be established. Nevertheless, this finding suggests that non-

glycosylated IGFBP-3 bound to VN may be a useful way to deliver IGF-I to sites where IGF-I is required to potentiate cell function such as in stimulating cell proliferation. Alternatively, non-glycosylated IGFBP-3 bound to VN may provide a mechanism for sequestering excess IGF-I in situations where cell proliferation is not required such as in tumours overexpressing IGFs.

#### EXAMPLE 6

***Competition binding assay assessing ability of IGF variants to compete with labelled IGFs for binding to VN in the presence of non-glycosylated recombinant IGFBP-3***

The concentration of IGFBP3 that produced the highest binding of radiolabelled IGF depicted in Figure 5 was used in a competition assay to determine whether increasing concentrations of IGFs and desIGFs could compete with radiolabelled IGFs for binding to vitronectin in the presence of IGFBP3. The results indicated that IGF-I, IGF-II, des(1-6)IGF-II and perhaps also des(1-3)IGF-I (not shown), at high concentrations, can compete with the binding of [<sup>125</sup>I]-IGF-II to vitronectin in the presence of 10 ng of IGFBP3 (FIG. 8). These experimental results indicated that only IGF-I,-II and des(1-6) IGF-II could compete with IGF-II for binding to vitronectin coated wells in the presence of 30 ng of IGFBP3.

#### EXAMPLE 7

***Stimulation of cell proliferation by isolated protein complexes comprising IGF-II and vitronectin***

The strategy of pre-binding IGF-II to VN was used in this study in an attempt to more accurately reflect the extracellular environment *in vivo*. Most cell culture approaches add exogenous substrates in solution phase; thereby the cells are constantly exposed to the treatments. Cells in tissues do not encounter this "constant, solution phase" environment *in vivo*. Thus the approach adopted for this study was to pre-bind IGFs to VN, a situation which more accurately mirrors the *in vivo* conditions.

Following pre-binding of IGFs to VN in culture dishes, cells were seeded into wells and the ability of IGFs complexed to VN to stimulate protein synthesis was examined by established methods (Francis *et al.*, 1986, Biochem J. 233:207-213). Increased protein synthesis correlates with increases in cell number, hence is a reflection of cell proliferation. Responses, expressed as percentage

above control wells in which no VN or IGFs were present, measured the incorporation of [ $^3\text{H}$ ]-leucine into newly synthesised protein over 48 hrs.

Referring to the data in FIG. 9, when 3, 10, 30, 100, 300 and 1000 ng of IGF-II was pre-bound to the wells in the absence of VN, resulting responses of 8, 12, 10, 16, 24 and 43% above the control wells (-VN, -IGF) respectively were observed. Furthermore, these same doses of IGF-II pre-bound to VN coated wells stimulated incorporation of [ $^3\text{H}$ ]-leucine into protein with effects of 19, 29, 39, 51, 70 and 101% respectively. Combining the responses obtained with VN alone (12%) with that obtained for IGF-II alone (refer to above) gives rise to predicted additive effects of 20, 24, 22, 28, 36 and 55% for 3, 10, 30, 100, 300 and 1000 ng of IGF-II respectively. These values are significantly different ( $p < 0.05$ ) to the actual effects observed when IGF-II was pre-bound to VN at all doses except for the two lowest amounts of IGF-II tested (3 and 10 ng). Thus IGF-II pre-bound to VN stimulates synergistic effects in protein synthesis ranging from 5 to 46% greater than the calculated additive effects. These responses may well be a result of the direct binding of IGF-II to VN, and may also arise from indirect binding of IGF-II to VN via IGFBPs. HaCAT keratinocytes produce large amounts of IGFBP-3 (Wraight *et al.*, 1994, J. Invest. Dermatol 103:627-631).

### EXAMPLE 8

#### ***Binding of labelled IGF-II to VN in the presence of recombinant IGFBPs produced in mammalian cells***

Binding assays examining the ability of [ $^{125}\text{I}$ ]-IGF-II to bind to VN-coated dishes in the presence of IGFBPs, added at the same time as the radiolabel, were performed as described in Upton *et al.*, 1999, *supra*. The IGFBPs used in these studies were glycosylated having been produced in mammalian cells. As shown in FIG. 10, increasing amounts of IGFBP-1, -3 and -6 resulted in decreased binding of labelled IGF-II to vitronectin coated wells in a dose-dependent manner. IGFBP-2 also appeared to compete for binding of labelled IGF-II to VN, albeit less effectively than IGFBP-1, -3 or -6. IGFBP-4 on the other hand had little effect on binding of IGF-II to VN, while IGFBP-5 appeared to enhance IGF-II binding to a small extent. The inhibitory effect of IGFBP-3 could result from IGFBP-3 competing for binding of IGF-II to the same binding region on VN. Alternatively,



or in addition, the inhibitory effect of IGFBP-3, as well as IGFBP-1 and -6. may arise from the affinity of IGF-II for VN being less than the affinity of IGF-II for these IGFBPs, hence these IGFBPs sequester IGF-II and the complex does not bind to VN.

5

#### EXAMPLE 9

##### *Stimulation of cell proliferation by isolated protein complexes comprising IGF-I, IGFBP-5 and vitronectin*

Referring to Table 2, IGF-I with IGFBP-5 and vitronectin stimulated keratinocyte proliferation (as measured by <sup>3</sup>H-leucine incorporation into newly synthesized protein) at all concentrations tested with synergistic effects observed at the highest amount tested.

10

It is proposed by the present inventors that the effect of isolated protein complexes upon cell proliferation may be even greater at higher concentrations of IGFBP-5 than the relatively low amount described in Table 2.

15

#### EXAMPLE 10

##### *Engineering IGF and IGFBP variants*

Amino acid residues in IGFBP-3 that are important for association with the ECM and have been defined as the putative "heparin-binding domain" are residues KGRKR at positions 228-232. Similar residues are found in the corresponding region of IGFBP-5. The IGFBP-3 HBD mutant that was used in the binding studies reported herein had the residues KGRKR altered to MDGEA based on the amino acids found in the corresponding positions in IGFBP-1. These changes result in a charge reversal in this part of the protein. This mutant still binds IGF-I and IGF-II with high affinity but binds the acid-labile subunit and the cell surface poorly (Firth *et al.*, 1998, J. Biol. Chem. **273** 2631-2638).

20

25

Heparin binding motifs in a diverse range of proteins were originally described in Cardin *et al.*, 1989, Arteriosclerosis **9** 21-32.

30

The C-domain of human IGF-II contains a number of positively charged amino acid residues and in particular positions 34-40 contains the amino acids RVSRRSR. Given that positively charged amino acids are important in mediating binding IGFBPs to cell surfaces and to VN, these amino acids in IGF-II may be important in binding of IGF-II directly to VN. Regardless, it would be a

relatively simple procedure to introduce a "heparin binding motif" similar to that found in IGFBP-3 (BXBBB; where B is a basic amino acid) by creating an IGF-II mutant with deletions of either V<sup>35</sup> or S<sup>36</sup> along with S<sup>39</sup>. The importance of positive residues in mediating binding of IGF-II to VN is further illustrated by the present  
 5 inventors' evidence of reduced binding of the chicken IGF-II mutant, (desR<sup>40</sup>)-IGF-II, to VN.

The C-domain of IGF-I on the other hand contains a relatively non-charged stretch of amino acids in the corresponding region of the protein to that described above for IGF-II. This may explain why IGF-I does not bind directly to  
 10 VN. In addition, insulin, which also does not bind to VN (or to IGFBPs) does not have a corresponding C-domain as it is cleaved out in the mature protein.

Human IGF-I            SSSRRAPQT

Human IGF-II        RVSRRS- - R

Introduction of the IGF-II sequence RVSRRSR or the IGFBP3  
 15 sequence KGRKR into IGF-I could enable IGF-I to bind VN directly.

### EXAMPLE 11

#### *Isolated protein complexes, cell proliferation and survival*

Bcl-2 transcription, a critical element of the cell survival pathway, is elevated in cells that attach to VN through alphav-beta3 integrins. (Matter & Ruoslahti, 2001,  
 20 J. Biol. Chem. **276** 27757-27763). In addition, IGF-I protects cells from apoptosis by elevating bcl-2 transcription in an AKT-dependent manner (Pugazhenthii *et al.*, 1999, J Biol. Chem. **274** 27529-35). The IGF receptor physically associates with the alphav-beta3 integrin with a synergistic effect on cell growth (Schneller *et al.*, 1997, EMBO J **16** 5600-5607). Thus isolated protein complexes of the  
 25 invention may provide an extracellular point of integration for initiating the cell survival signals mediated by both the integrin and the growth factor receptor.

IGFBP-5 has been demonstrated to potentiate the anti-apoptotic and mitogenic effects of IGF-I in prostate cancer cells (Miyake *et al.*, 2000, Endocrinol. **141** 2257-2265). In addition, the synthesis of VN *in vivo* by glioma  
 30 cells and in colorectal adenocarcinoma correlates with tumour grade (Uhm *et al.*, 1999, Clin Cancer Res. **5** 1587-1594; Tomasini-Johansson *et al.*, 1994, Exp Cell Res. **214** 303-312; Gladson *et al.*, 1995, J. Cell Sci. **108** 947-56; Gladson &

Cheresh, 1991, J. Clin. Invest. **88** 1924-32).

Hence, according to the present invention, it is proposed that IGF:IGFBP:VN complexes formed *in vivo* may promote tumour cell survival and progression. The invention therefore contemplates therapeutic agents that disrupt *in vivo* complex formation.

The heparin-binding domain of VN has been reported to inhibit fibronectin matrix assembly (Hocking *et al.*, 1999, J. Biol. Chem. **274** 27257-27264). Reduced fibronectin deposition is associated with tumour cell invasion as decreased cell migration rates are associated with increased levels of polymerised fibronectin (Morla *et al.*, 1994, Nature **367** 193-196). Hence, IGF:IGFBP complexes bound to the heparin binding domain of VN, may dampen fibronectin matrix assembly and facilitate tumour invasion of local connective tissue. Thus, the invention contemplates therapeutic agents that disrupt these *in vivo* complexes to reduce tumour invasiveness.

#### ***Isolated protein complexes and wound healing***

The converse argument can be used to support the use of the complex in situations where cell migration is required such as in wound repair. The IGF system plays an important role in wound healing and both IGF-I and IGFBP-3 are present in wound fluid in significant concentrations. (Skottner *et al.*, 1990, Acta Scand. Suppl. **367** 63-66; Clark R (ed) 1996, Molecular and Cell Biology of Wound Repair, pp 3-50, Plenum Press, New York; Robertson *et al.*, 1996, Endocrinol. **137** 2774-2784; Vogt *et al.*, 1998, Growth Horm. IGF Res. **8** Suppl B:107-9.

IGFBPs have been demonstrated to reduce the rate of IGF clearance from wounds. (Robertson *et al.*, 1999, Am J Physiol. **276** E663-71).

IGFBP-3:IGF-I complexes bind to fibrin clots *in vitro* leading to the suggestion this also occurs *in vivo*, resulting in concentration of IGF-I at wound sites. (Campbell *et al.*, 1999, J. Biol. Chem. **274** 30215-30221). Similarly, vitronectin binds to fibrin (Podor *et al.*, 2000, J. Biol. Chem. **275** 19788-19794). It is also noted that vitronectin-null mice exhibit increased wound fibrinolysis and decreased microvascular angiogenesis (Jang *et al.*, 2000, Surgery **127** 696-704).

According to the present invention, it is proposed that IGFs bound to IGFBPs can bind to VN, which in turn associates with the fibrin clot, thus

providing a reservoir of IGFs at the wound site. Thus isolated protein complexes of the invention could be administered to wounds to accelerate the repair process.

A particular aspect of wound healing contemplated by the present invention relates to healing diabetic foot ulcers. Wound healing is delayed in diabetes. Growth factors influence the healing process and in particular, IGFs have been shown to stimulate keratinocyte proliferation. However, analysis of tissues from diabetic skin and foot ulcers reveals lack of expression of IGF-I within the basal layer and fibroblasts compared to tissue sections from non-diabetic patients. (Blakytyn *et al.*, 2000, *J. Pathol.* **190** 589-594). Isolated protein complexes of the invention could be useful in delivery of IGF-I to these types of wounds.

#### *Isolated protein complexes and bone engineering*

IGFBP-5 facilitates binding of labelled IGF-I to bone by a mechanism that is independent of IGF receptors. (Mohan *et al.*, 1995, *J. Biol. Chem.* **270** 20424-20431) and enhances IGF-stimulated osteoblast function. (Andress, 1995, *J. Biol. Chem.* **270** 28289-28296).

The implant material hydroxylapatite has been shown in numerous studies to be highly biocompatible and to osseointegrate well with existing bone. Recent evidence has found that hydroxylapatite will absorb more VN from serum than other commonly used implant materials such as titanium and stainless steel. The absorption of VN was accompanied by greater binding of osteoblast precursor cells (Kilpadi *et al.*, 2001, *J. Biomed. Mater. Res.* **57** 258-267).

The effect of VN on nanophase alumina vs conventional alumina was recently examined with VN being found to enhance osteoblast adhesion (Webster *et al.*, 2001, *Tissue Eng* **7** 291-301).

The present inventors propose that isolated protein complexes of the invention may be useful as coating applied to these materials and thereby accelerate bone cell attachment, growth and integration in orthopaedic applications such as hip joint replacements.

VN enhances IGF-I stimulated osteoclastic resorption and proteinase activities in rabbit bone cell culture. (Rousselle *et al.*, 2001, *Histology & Histopathology* **16** 727-734) and IGFBP-5 enhances IGF-stimulated osteoblast mitogenesis. (Andress & Birnbaum, 1992, *J. Biol. Chem.* **267** 22467-22472).

The present inventors propose that as the major IGFBP produced by bone cells is IGFBP-5, the potentiation of the IGF effect by VN is likely to also involve IGFBP-5.

*Isolated protein complexes and treatment of atherosclerosis*

5 IGF-I has previously been implicated in the development of experimental atherosclerotic lesions. Moreover, alphav-beta3 inhibitors have been demonstrated to reduce atherosclerotic lesions - this being associated with inhibition of IGF-I-mediated signaling. (Nichols *et al.*, 1999, Circ. Res. **85**1040-1045).

Given that:

10 (i) IGFBP-5 and VN are synthesised and secreted by arterial smooth muscle cells and are present in blood vessel walls; and

(ii) IGF:IGFBP-5:VN complexes promote vascular smooth muscle cell mitogenesis and migration (Nam & Clemmons, 2000, Growth Horm. IGF Res. **10**:A23);

15 it is proposed by the present inventors that IGF:IGFBP-5:VN complexes are involved in formation of atherosclerotic lesions. Thus therapeutic agents that disrupt complex formation may hold potential in treating atherosclerosis.

Throughout the specification the aim has been to describe the preferred embodiments of the invention without limiting the invention to any one  
20 embodiment or specific collection of features. It will therefore be appreciated by those of skill in the art that, in light of the instant disclosure, various modifications and changes can be made in the particular embodiments exemplified without departing from the scope of the present invention.

It will also be appreciated that all patent and scientific literature  
25 and computer programs referred to are incorporated herein by reference.

TABLE 1

Nucleic acid	Reference
IGF-I	Jansen <i>et al.</i> , 1983, Nature <b>306</b> 609
IGF-II	Jansen <i>et al.</i> , 1985, FEBS Lett <b>179</b> 243
IGFBP-1	Brinkman <i>et al.</i> , 1988, EMBO J. <b>7</b> 2417
IGFBP-2	Binkert <i>et al.</i> , 1989, EMBO J. <b>8</b> 2497
IGFBP-3	Wood <i>et al.</i> , 1988, Mol. Endocrinol. <b>2</b> 1176
IGFBP-4	LaTour <i>et al.</i> , 1990, Mol. Endocrinol. <b>4</b> 1806
IGFBP-5	Kiefer <i>et al.</i> , 1991, Biochem. Biophys. Res. Comm. <b>176</b> 219
IGFBP-6	Shimasaki <i>et al.</i> , 1991, Mol. Endocrinol. <b>5</b> 938
ALS	Leong <i>et al.</i> , 1992, Mol. Endocrinol. <b>6</b> 870
Vitronectin	Suzuki <i>et al.</i> , 1985, EMBO J. <b>4</b> 2519

TABLE 2

Treatment	- Vitronectin	+ Vitronectin
Control	100 ± 2.6	-
IGFBP-5	-	109.5 ± 9.2
100 ng IGF-1 + IGFBP-5	117.4 ± 10.2	119.4 ± 1.9
300 ng IGF-1 + IGFBP-5	140.9 ± 2.0	154.3 ± 1.8
1000 ng IGF-1 + IGFBP-5	144.3 ± 11.0	161.4 ± 9.1

CLAIMS

1. An isolated protein complex comprising a growth factor binding protein and vitronectin.
2. The isolated protein complex of Claim 1, further comprising a growth factor.
3. The isolated protein complex of Claim 2, wherein the growth factor is insulin like growth factor-I (IGF-I).
4. The isolated protein of Claim 2, wherein the growth factor is selected from the group consisting of epidermal growth factor (EGF), fibroblast growth factor (FGF), basic fibroblast growth factor (bFGF), osteopontin, thrombospondin-1, tenascin-C, PAI-1, plasminogen, fibrinogen, fibrin and transferrin.
5. The isolated protein complex of Claim 1, wherein the growth factor binding protein is selected from the group consisting of IGFBP1, IGFBP2, IGFBP3, IGFBP4, IGFBP5 and IGFBP6.
6. The isolated protein complex of Claim 5, wherein the insulin-like growth factor binding protein is IGFBP2, IGFBP3, IGFBP4 or IGFBP5.
7. The isolated protein complex of Claim 6, wherein the insulin-like growth factor binding protein is IGFBP3 or IGFBP5.
8. An isolated protein complex comprising an IGFBP-related protein and vitronectin.
9. The isolated protein complex of Claim 8, further comprising a growth factor.
10. This isolated protein complex of Claim 9, wherein the growth factor is IGF-I or IGF-II.
11. The isolated protein complex of Claim 10, wherein the IGFBP-related protein is selected from the group consisting of:
  - (i) connective tissue growth factor (CTGF);
  - (ii) a polypeptide encoded by the *mac25* gene;
  - (iii) a polypeptide encoded by the *nov* gene; and
  - (iv) a polypeptide encoded by the *cyr61* gene.
12. An isolated protein complex comprising vitronectin, a variant growth factor and/or a variant growth factor binding protein.

13. The isolated protein complex of Claim 12, comprising vitronectin and a variant growth factor engineered to include a heparin binding domain (HBD).
14. The isolated protein complex of Claim 13, wherein the variant growth factor is IGF-I engineered to include said HBD.
- 5 15. The isolated protein complex of Claim 15, consisting of IGF-I engineered to include said HBD bound directly to vitronectin.
16. The isolated protein complex of Claim 13, wherein the HBD has the amino acid sequence BXBBB, wherein B is a basic amino acid residue.
17. The isolated protein complex of Claim 14, wherein the HBD has the amino acid sequence KGRKR.
- 10 18. A variant IGFBP having a deleted or mutated HBD, wherein the IGFBP is unable to form the isolated protein complex of Claim 1.
19. The variant IGFBP of Claim 19 which is capable of binding IGF-I.
20. Use of the variant of Claim 19 as an antagonist.
- 15 21. The isolated protein complex of Claim 12, comprising vitronectin and a non-glycosylated growth factor binding protein.
22. The isolated protein complex of Claim 21, wherein the non-glycosylated growth factor binding protein is IGFBP3.
23. The isolated protein complex of Claim 13, comprising vitronectin and a variant growth factor selected from the group consisting of des(1-6)IGF-II and des(1-3)IGF-I.
- 20 24. The isolated protein complex of Claim 1, Claim 8 or Claim 12, wherein vitronectin is multimeric.
- 25 25. The isolated protein complex of Claim 1, Claim 8 or Claim 12. wherein vitronectin is monomeric.
26. The isolated protein complex of Claim 24, wherein each vitronectin monomer is bound to the same or different IGFBP selected from the group consisting of: IGFBP1, IGFBP2, IGFBP3, IGFBP4, IGFBP5 and IGFBP6.
27. The isolated protein complex of Claim 26, further comprising IGF-II directly bound to vitronectin.
- 30 28. A pharmaceutical composition comprising the isolated protein complex of any one of Claims 1, 8 or 12 and a pharmaceutically-acceptable carrier or diluent.



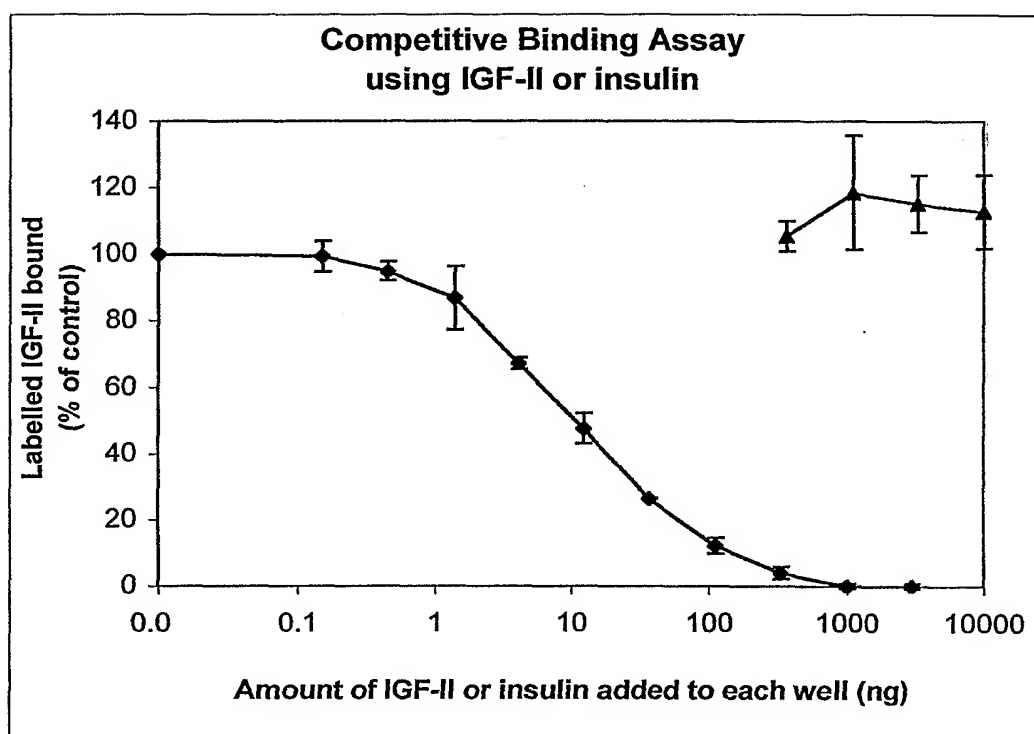
29. A surgical implant or prosthesis comprising the isolated protein complex of any one of Claims 1, 8, or 12.
30. A transformed cell capable of expressing a recombinant protein complex according to any one of Claims 1, 8 or 12, or recombinant polypeptides capable of forming said complex.
31. A method of modulating cell proliferation and/or migration including the step of administering to an animal or isolated cells thereof, an isolated protein complex according to any one of Claims 1, 8 or 12.
32. A method of modulating cell proliferation and/or migration, including the step of administering to an animal or isolated cells thereof an agent which prevents or disrupts formation of the protein complex of any one of Claims 1, 8 or 12.
33. The method of Claim 32 wherein the agent prevents or disrupts an interaction between an IGFBP and vitronectin.
34. The method of Claim 32, wherein the agent is IGF-II.
35. Use of IGF-II to inhibit formation of the isolated protein complex of Claim 1.
36. Use of the isolated protein complex of any one of Claims 1, 8 or 12 for the preparation of a medicament for the treatment of an epithelial cell disease.
37. Use according to Claim 36 wherein the epithelial cell disease is psoriasis or epithelial breast cancer.
38. Use according to Claim 36, wherein the epithelial cell disease causes impaired gut function through deterioration of the gastrointestinal epithelial lining.
39. Use of the isolated protein complex of any one of Claims 1, 8 or 12 for the preparation of a medicament for promoting wound healing, skin repair, ulcer and burn healing or *in vitro* skin regeneration.
40. Use of the isolated protein complex of any one of Claims 1, 8 or 12 for the preparation of a medicament for promoting bone regeneration.
41. Use of the isolated protein complex of any one of Claims 1, 8 or 12 for the preparation of a medicament for promoting repair of damaged neuronal tissue.
42. Use of an agent that disrupts or prevents formation of the isolated protein complex of any one of Claims 1, 8 or 12 for the preparation of a medicament for

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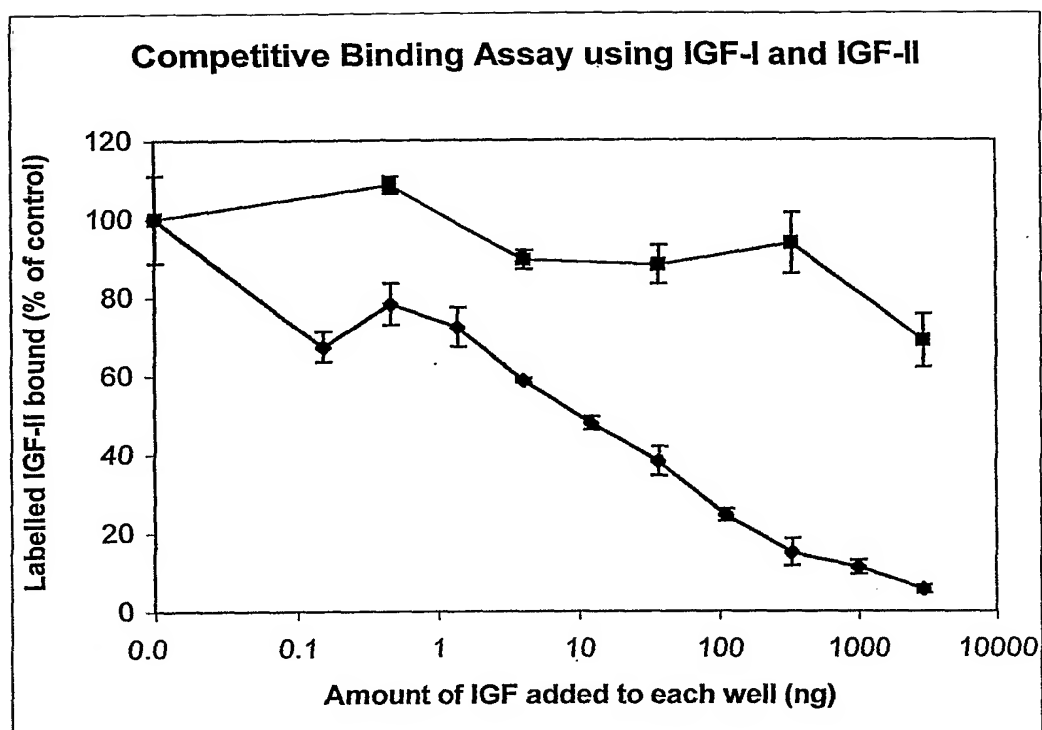
treating cancer.

43. Use of an agent that disrupts or prevents formation of the isolated protein complex of any one of Claims 1, 8 or 12 for the preparation of a medicament for treating atherosclerosis.

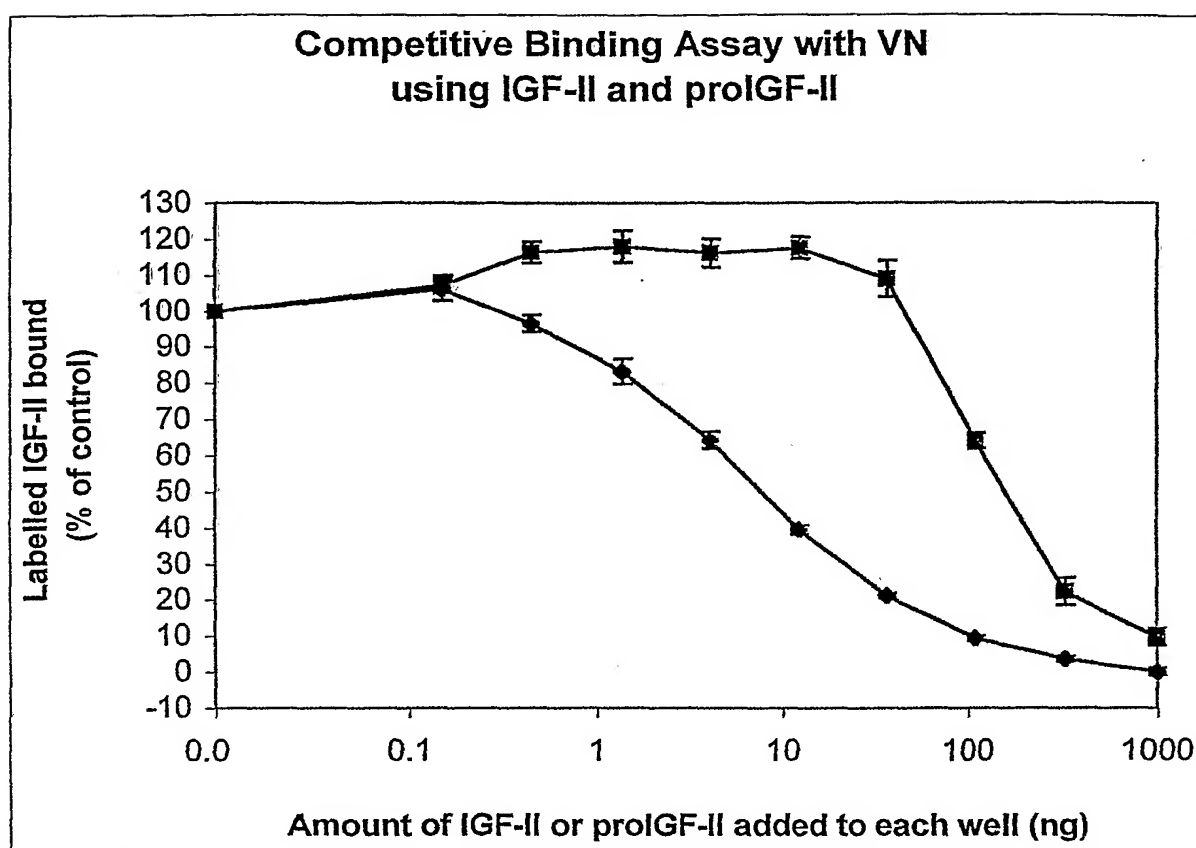
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**FIG. 1**

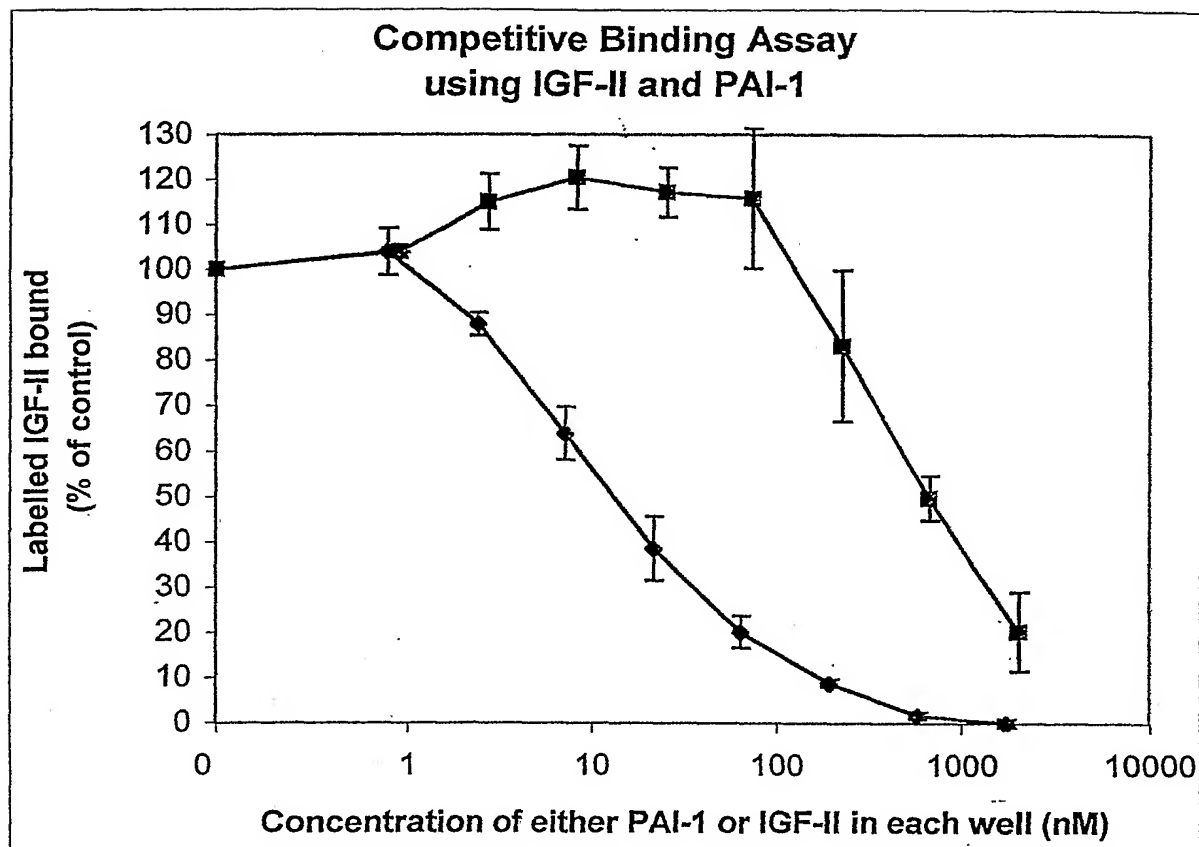
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**FIG. 2**

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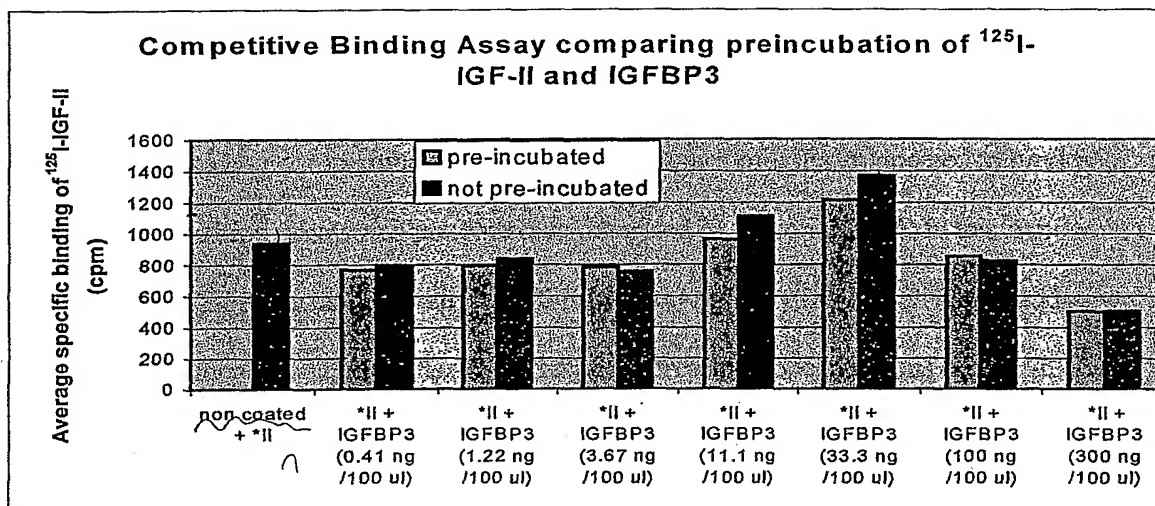
**FIG. 3**

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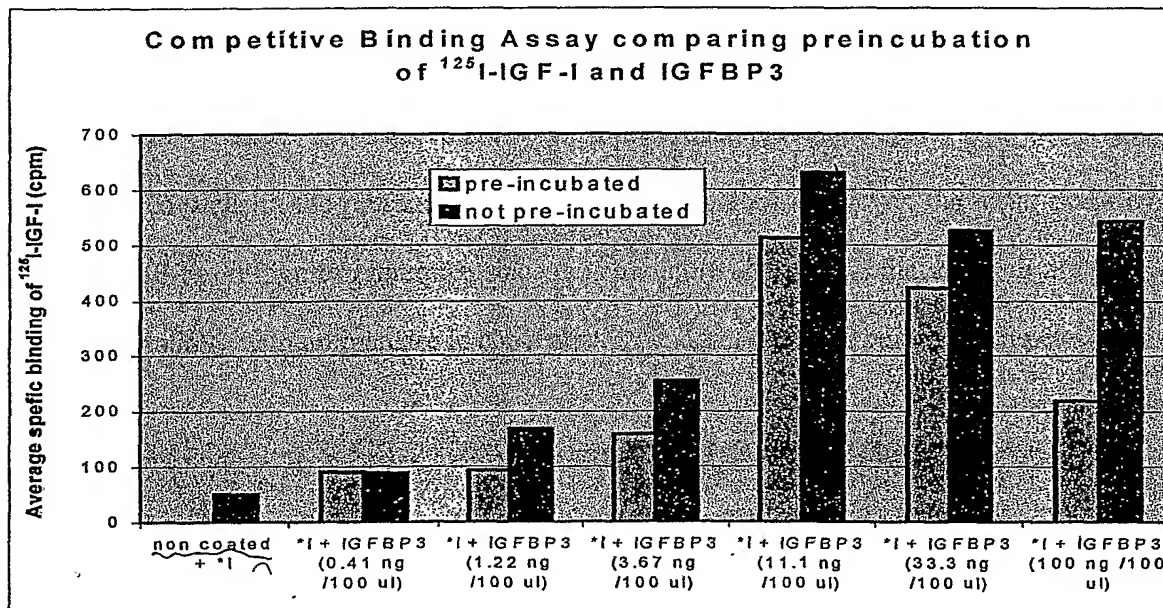
**FIG. 4**

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(a)

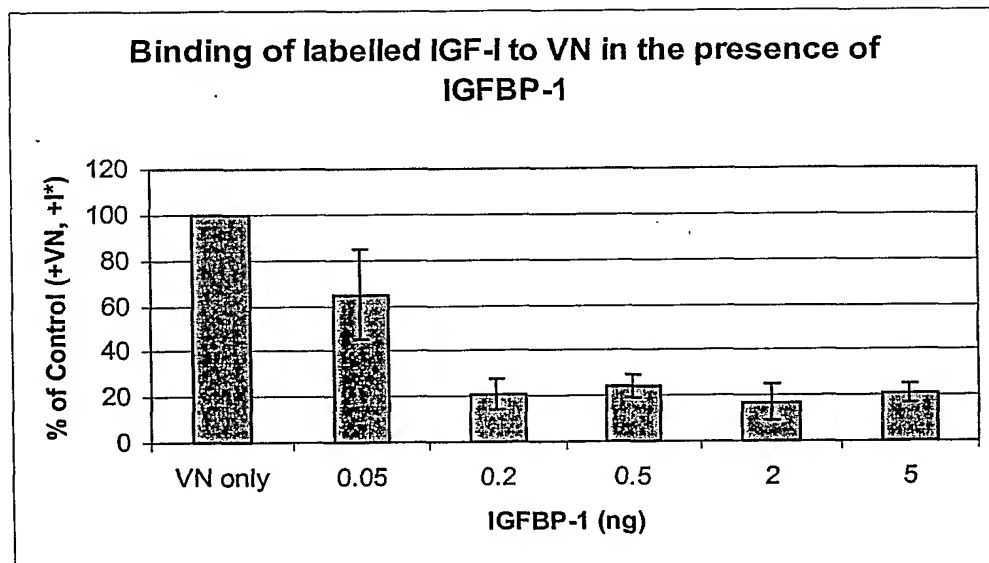


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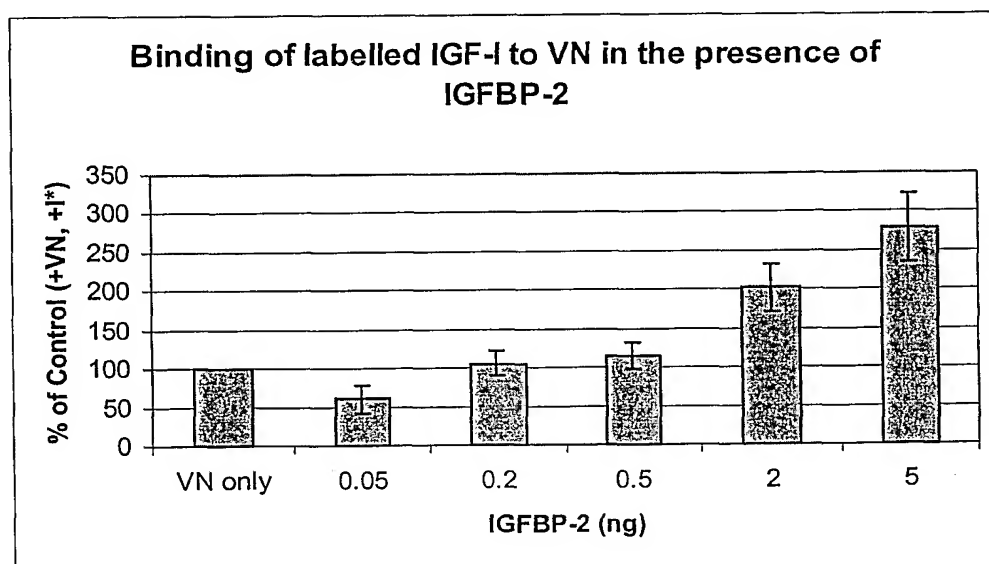
**FIG. 5**

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A



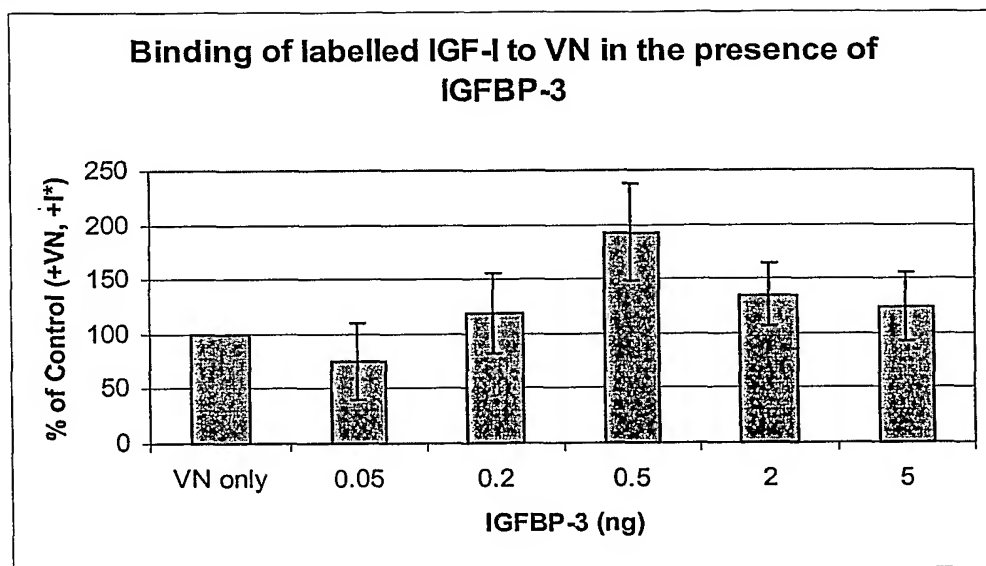
B

**FIG. 6**

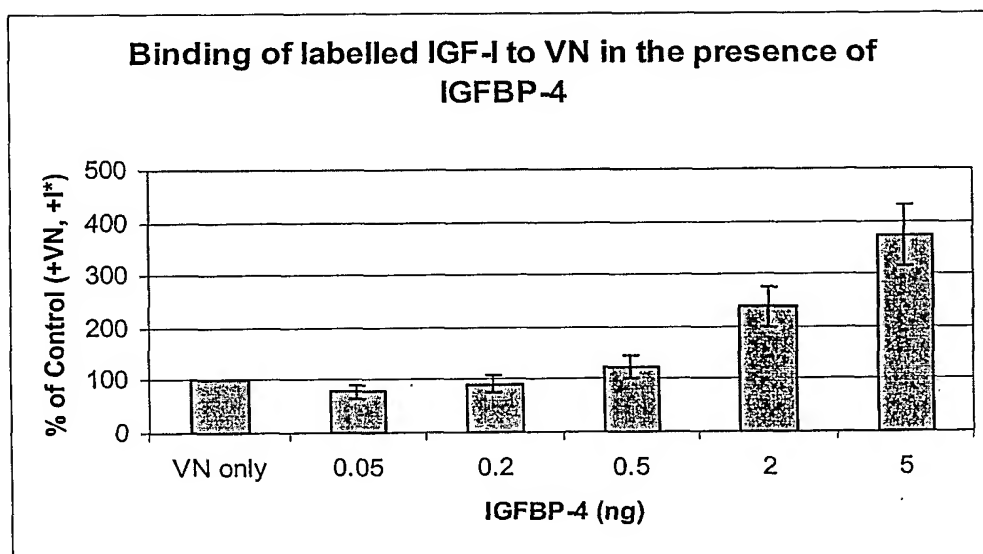


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C

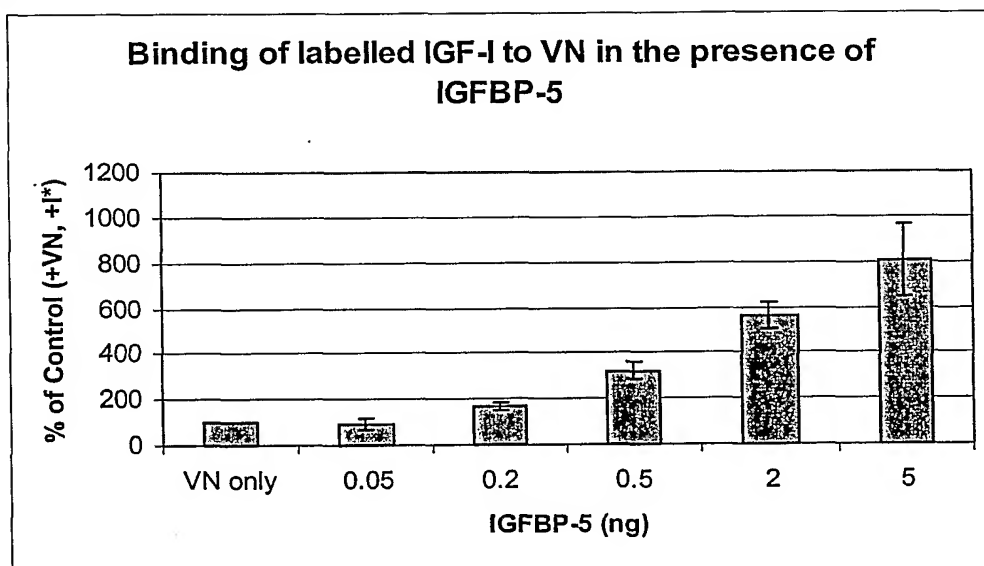


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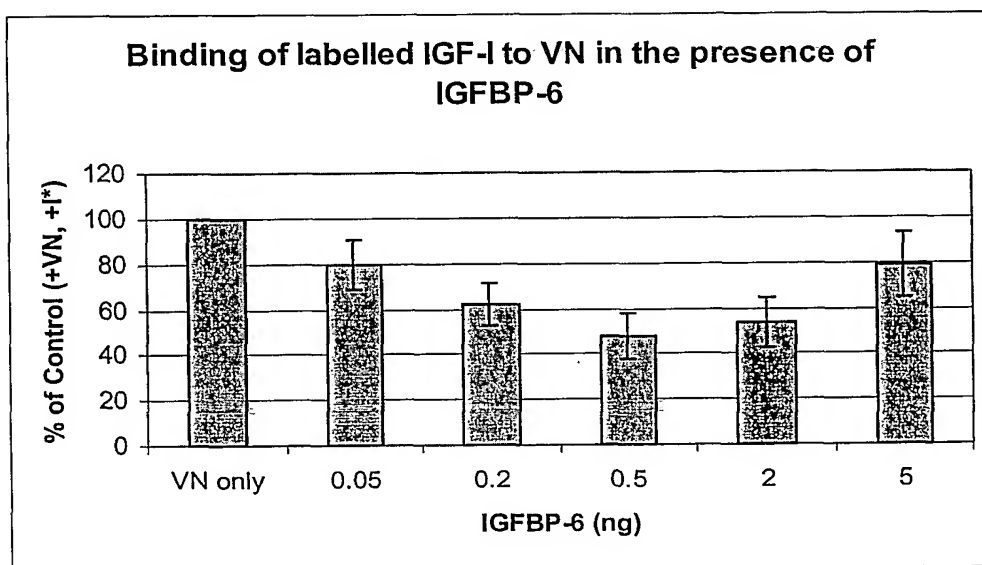
**FIG. 6**

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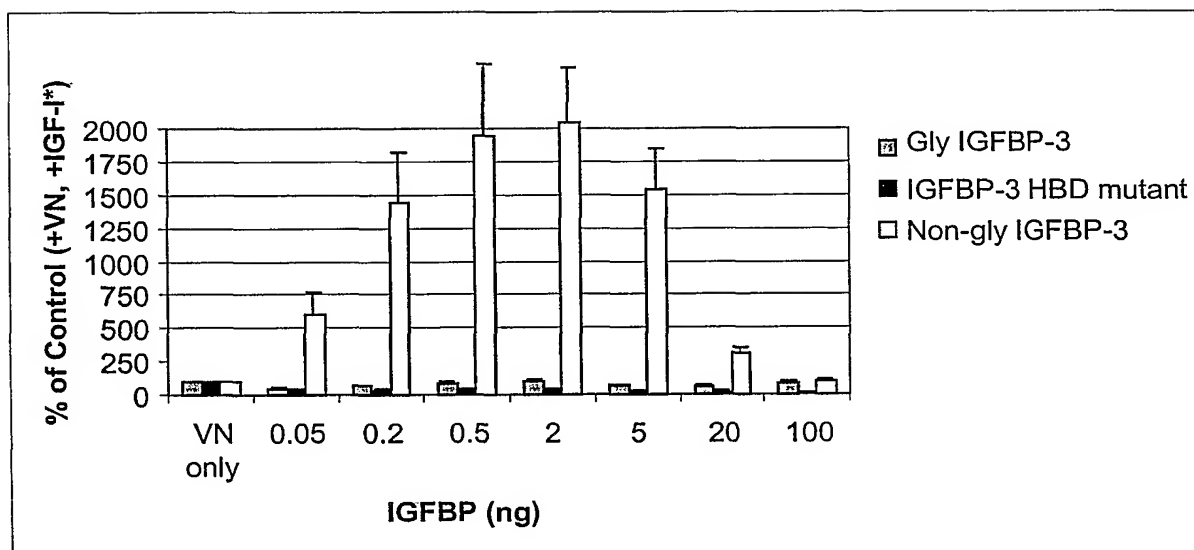
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F

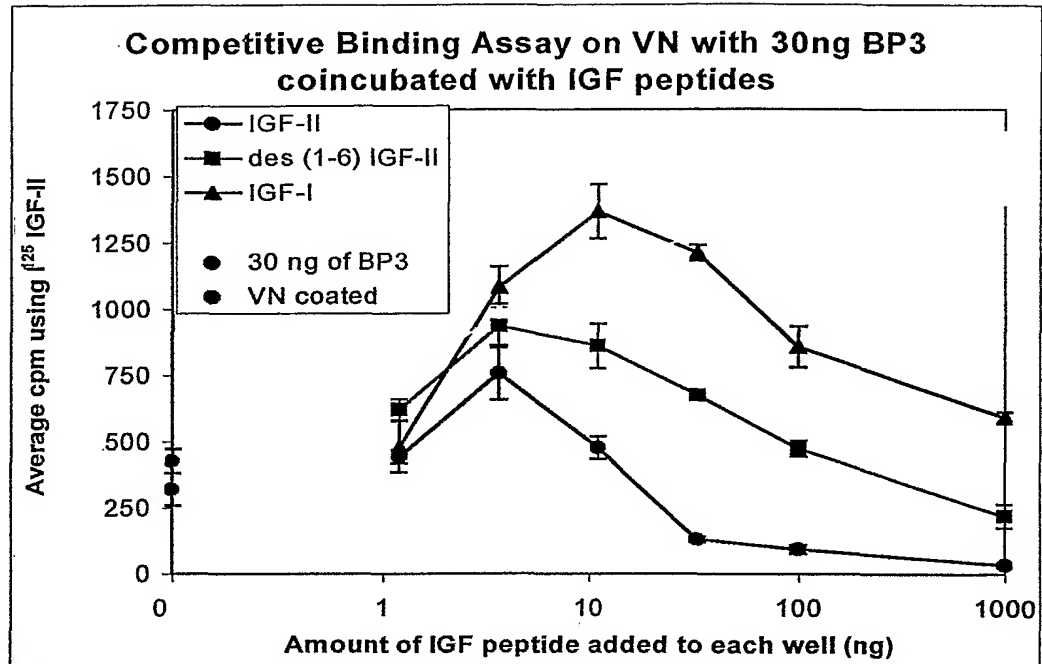
**FIG. 6**

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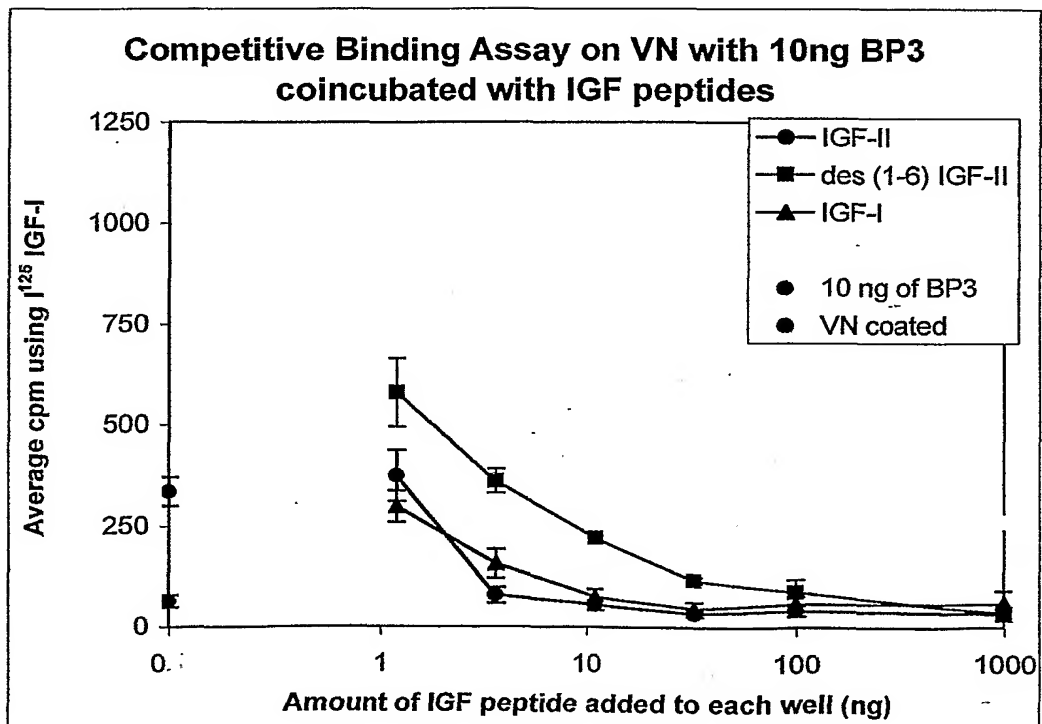
**FIG. 7**

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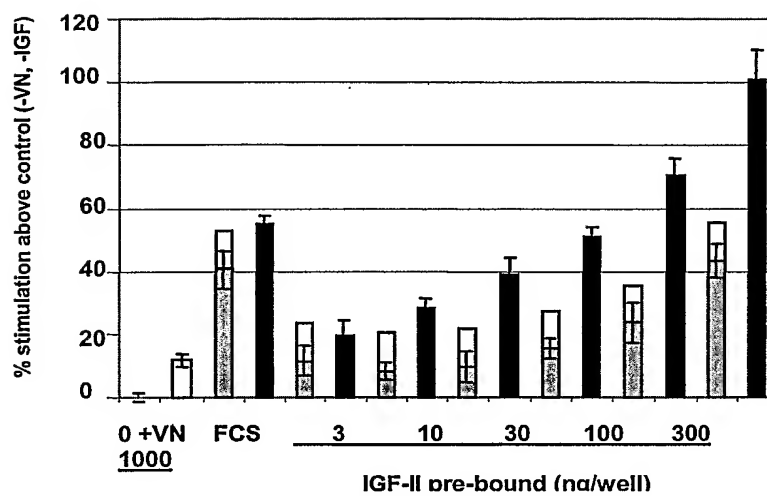
(a)



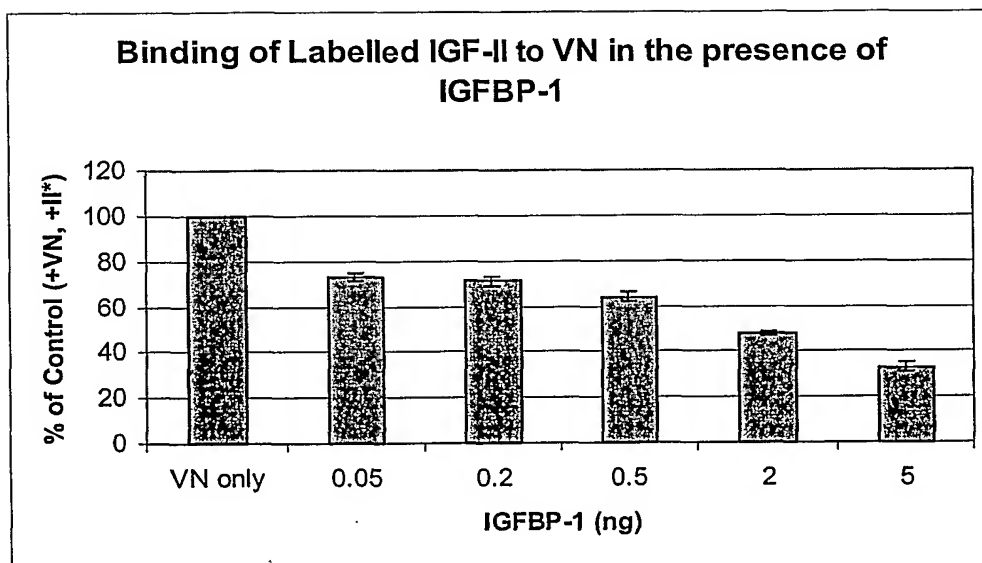
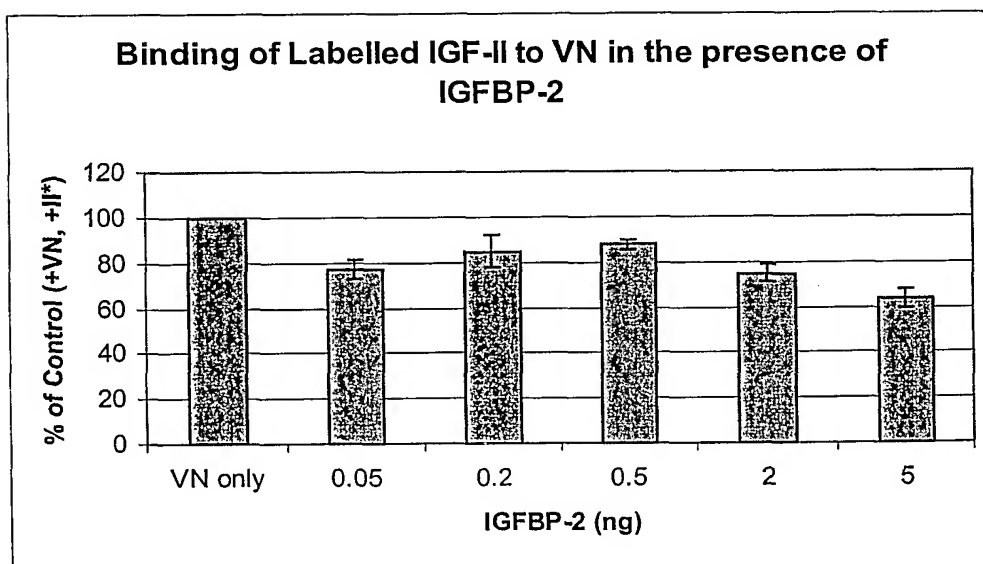
(b)

**FIG. 8**

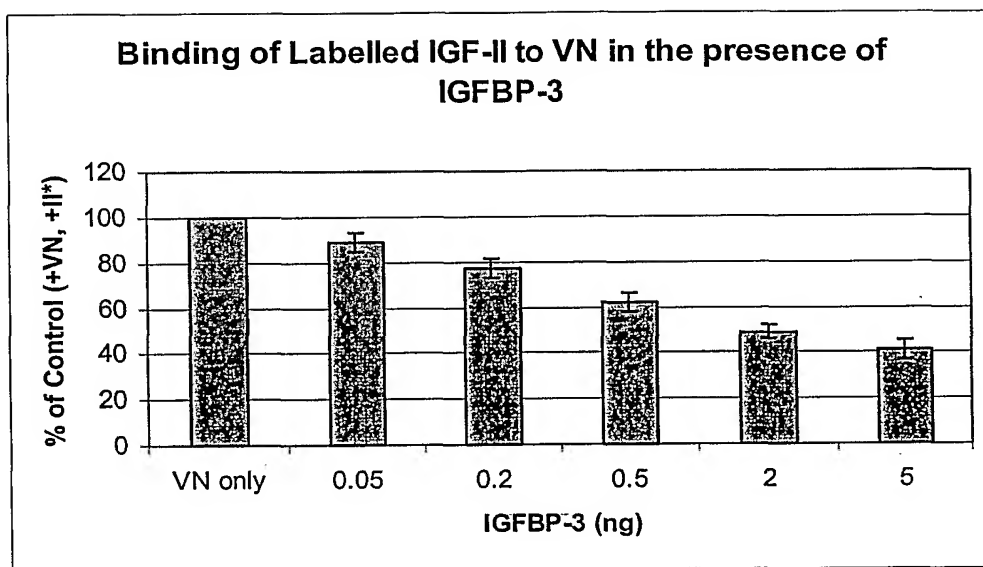
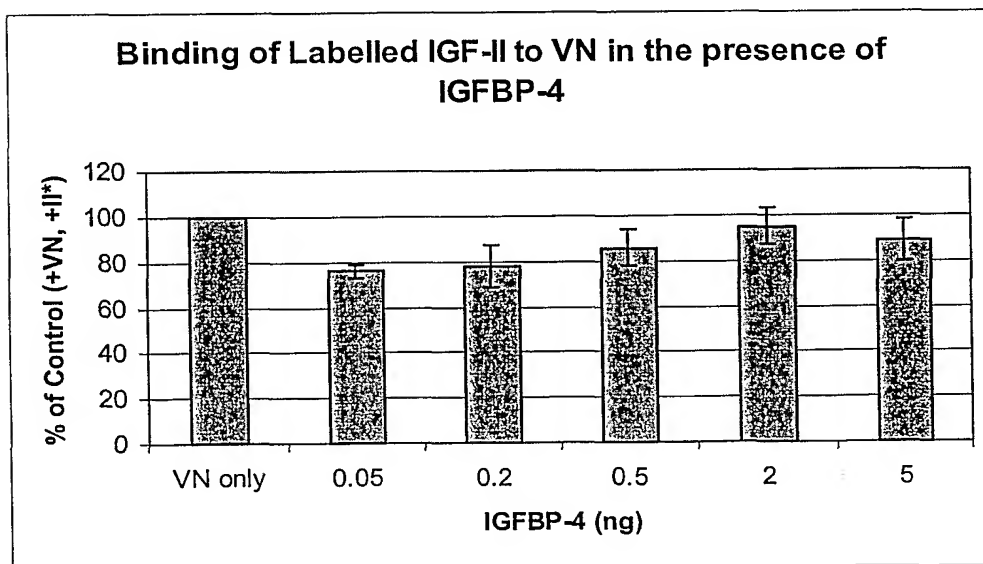
11/14

**FIG. 9**

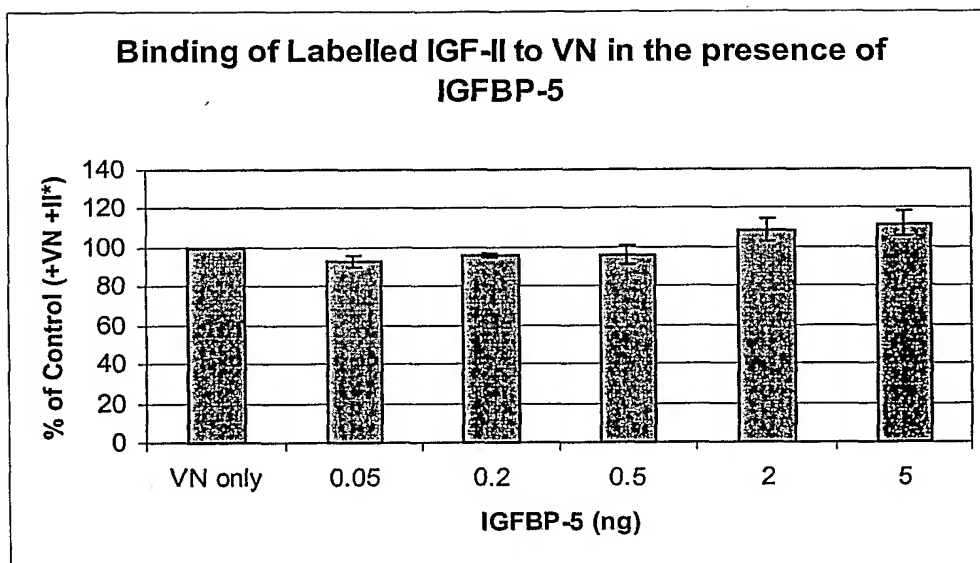
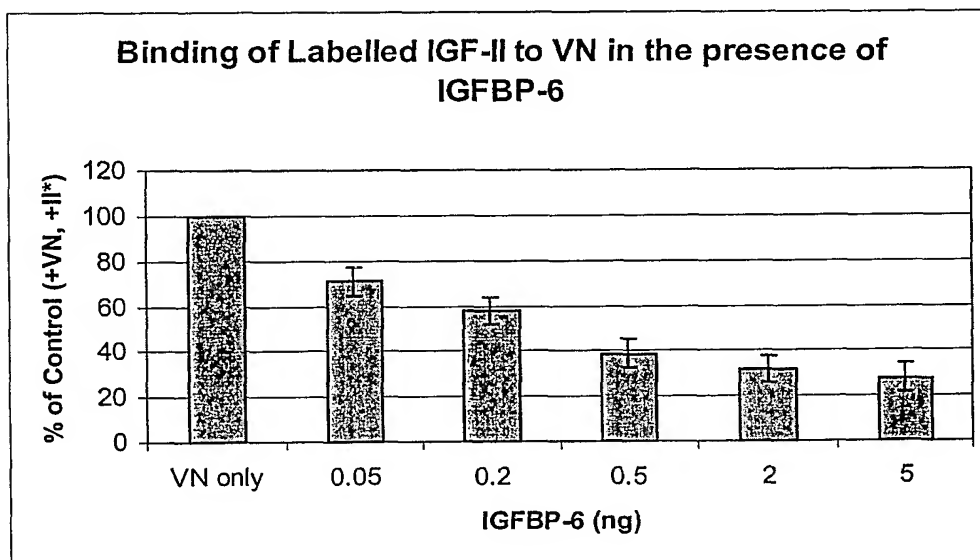
12/14

**A****B****FIG. 10**

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**C****D****FIG. 10**

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**E****F****FIG. 10**



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU01/01196

**A. CLASSIFICATION OF SUBJECT MATTER**Int. Cl. <sup>7</sup>: A61K 38/39, 38/27, 38/30, 38/31, 38/39 A61P 19/04, 1/00, 5/00, 37/00, 9/10, 19/10,

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DWPI/MEDLINE: vitronectin and growth factor and growth factor binding protein and proliferation and migration and similar terms.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	WO-A-00/55206 (The Hope Heart Institute) 21 September 2000	1-17
PY	see whole document	1-43
X	Klemke, R. L. et al "Receptor Tyrosine Kinase Signaling Required for Integrin $\alpha v \beta 5$ -directed Cell Motility but Not Adhesion on Vitronectin". J. Cell Biol. 1994 127/3 pages 859-866.	1-17, 21-43
Y	See Discussion	1-43

☒ Further documents are listed in the continuation of Box C
 ☒ See patent family annex

* Special categories of cited documents:		"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier application or patent but published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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"P"	document published prior to the international filing date but later than the priority date claimed		

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU01/01196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Loskutoff, D et al "Regulation of cell adhesion by PAI-1". APMIS, 1999, Vol 107 pages 54-61. See pages 54, 55, 57	1, 2, 4, 12, 28, 31-43
Y		1-43
X	Firth, S et al. "Structural Determinants of Ligand and Cell Surface Binding of Insulin-like Growth Factor-binding Protein 3". J. Biol. Chem., 1998, 273/5 pages 2631-2638 See whole document	18-20
X	Jones, J. I. et al, "Ligand occupancy of the $\alpha V\beta 3$ integrin is necessary for smooth muscle cells to migrate in response to insulin-like growth factor I". Proc. Nat. Acad. Sci. USA, 1996 Vol 93 pages 2482-2487 See whole document	1-17, 21-43
Y		1-43
X	Jones, J. I. et al, "Cell Migration: Interactions among Integrins, IGFs and IGFBPs". Progress in Growth Factor Res., 1995 6/2-4 pages 319-327 See whole document	1-17, 21-43
Y		1-43
X	Rouselle A. et al. "Potential synergies between matrix proteins and soluble factors on resorption and proteinase activities of rabbit bone cells". Hist. And Histopath., July 2001 Vol 16 pages 727-734 See page 733	1-17, 40
X	Upton, Z et al "Identification of vitronectin as a novel insulin-like growth factor-II binding protein". Endocrinology 1999 140/6 pages 2928-2931. See Abstract and page 2930	12, 23, 24, 25, 28
Y		1-43
Y	Taraboletti, G. et al, "The 140-Kilodalton Antiangiogenic Fragment of Thrombospondin-1 Binds to Basic Fibroblast growth Factor". Cell Growth and Differentiation, 1997 Vol 8 pages 471-479 See whole document	1-43
X	Mousa, S et al "Role of Hypoxia and Extracellular Matrix-Integrin Binding in the Modulation of Angiogenic Growth Factors Secretion by Retinal Pigmented Epithelial Cells". J. Cellul. Biochem., 1999 74/1 pages 135-143	1, 2, 4, 12, 28, 31, 32, 36
Y		1-43

**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

International application No.  
**PCT/AU01/01196**

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member	
WO	200055206	AU	200038972
END OF ANNEX			